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SCIENTIFIC EDITION

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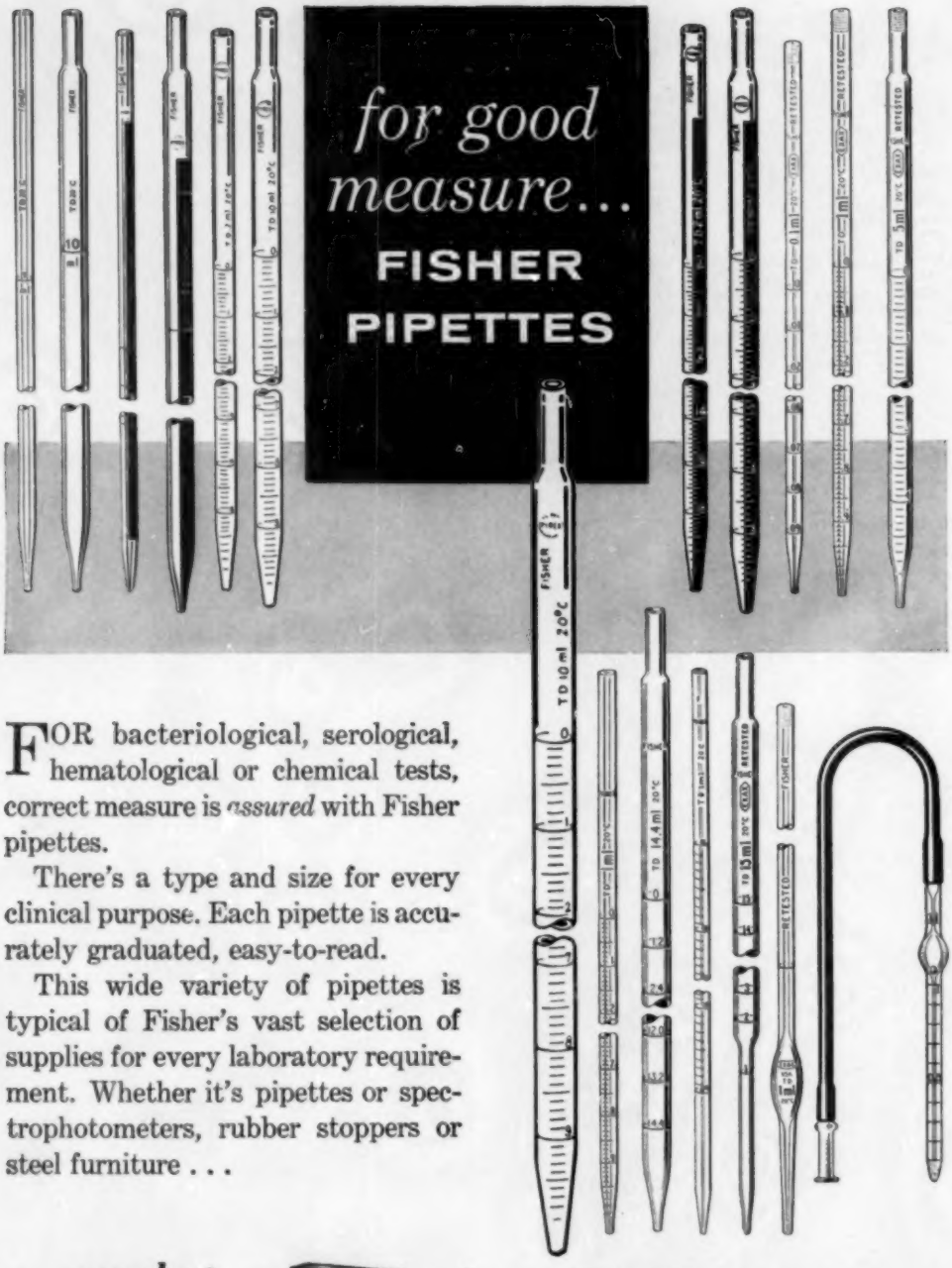

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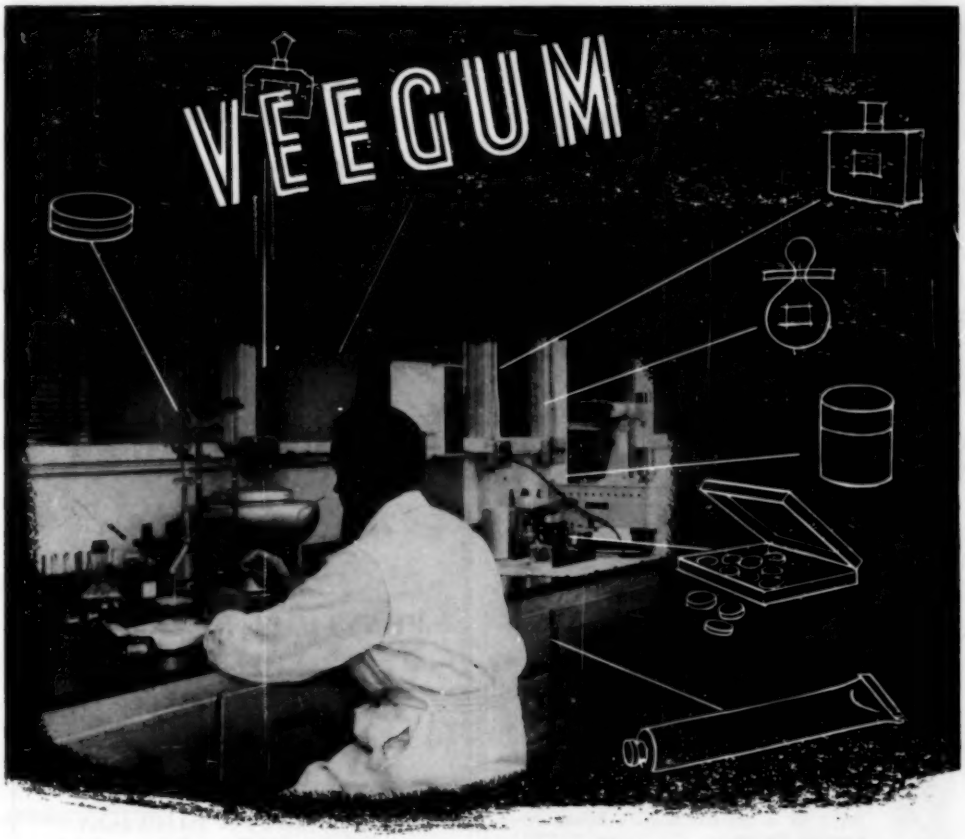

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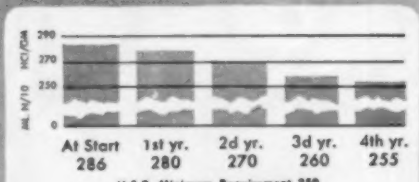

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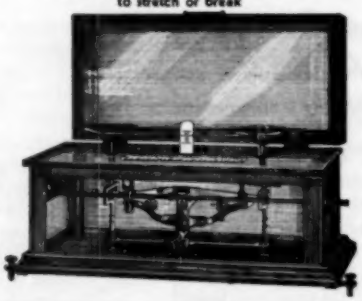

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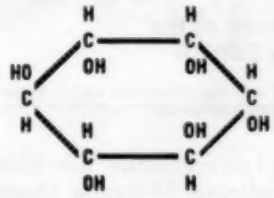

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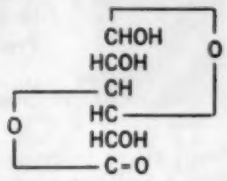

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(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▶ The inside front cover is given over to **Abbott's SUR-BEX** (B-complex Tablets with C), and in another two-page insertion, the manufacturer reports on the efficacy of its antibiotic, *filmtab* **Erythrocin Stearate.**

▶ Pharmacists may well pay heed to the question posed by **The American Druggists' Insurance Company:** "Is your fire insurance adequate to protect the increased value of your property. . .?"

▶ The **Ames Company** also queries the reader: "Have you noticed," it asks, "how many customers ask for . . . **Clinitest** *Sealed-in-Foil* Reagent Tablets?" Recommended for urine-sugar testing, each tablet is individually foil-wrapped to protect it against moisture or careless handling.

▶ Available through wholesalers or the Colgate-Palmolive Co. is a *new one inch size* **Vaseline** gauze strip manufactured by the *Professional Products Division* of the **Chesebrough Mfg. Co.**

▶ For use in the treatment of diarrhea, the **Hynson, Westcott & Dunning** message is devoted to describing the effectiveness of **Lactinex Granules** and **Tablets.**

▶ "The leading line of tetracycline" is **Lederle's** way of describing **Achromycin S.F.,** its broad-spectrum antibiotic, compounded with the *stress vitamin formula suggested by the National Research Council.*

▶ In announcing its new nonbarbiturate sedative, **Eli Lilly and Company** tags **'Valmid'** with the apposition, "the touch of sleep." It is recommended as being useful in simple insomnia when barbiturates are contraindicated.

▶ The **S. E. Massengill Company** advocates the maintenance of adequate stocks of its prescription specialties: **Adrenosem, Aminodrox, Gallogen, Livitamin, Massengill Powder, Obedrin, Salcort** and **Semhyten.**

▶ In a pertinent and timely announcement, **Parke, Davis & Company** observes that **Ziradryl Cream** "is *compounded specifically* for prevention and treatment of poison ivy and poison oak dermatitis."

▶ The **Pfizer Laboratories'** message makes news with a *first:* "News!" it reads, "The *first chewing gum* that prevents motion sickness of all types—**Bonamine** *Chewing Tablets.*"

▶ The **A. H. Robins Company's** contribution for the treatment of diarrhea is **Donnagel.** Containing kaolin and pectin compound, it provides "comprehensive" therapeutic control of the major factors causing diarrhea.

▶ In its continuing series of **Benzedrex Inhaler** promotions, **Smith, Kline & French Laboratories** adds to the long list of testimonials subscribing to the merchandising effectiveness of its display carton.

▶ The **Warner-Chilcott** announcement introduces new sizes and forms of established products—**Peritrate,** effective prophylaxis against angina pectoris, and **Methium,** "the most potent hypotensive agent, now enhanced in usefulness for wider indications."

▶ For "hospital clean" skin, **Winthrop-Stearns** is marketing a creamy, white emulsion, **pHisoHex.** Bottled in a refillable "squeeze" bottle, it is recommended for cleansing infected skin areas, for bathing infants and for general sick room use.

▶ The **Wyeth** insertion stresses the high profit margin with **Collyrium,** "America's Favorite Eye Lotion."

Look for details in the Practical Pharmacy Edition of THIS JOURNAL*—Out June 20*

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIV — *JUNE, 1955* — NUMBER 6 — CONSECUTIVE NO. 12

A Study of Carbon^{14} Labeled Acetate in *Digitalis Purpurea* Glycoside Biosynthesis*

By E. H. DJAO and H. W. YOUNGKEN, JR.

A study has been made of the incorporation of acetate carboxyl carbon^{14} by *Digitalis purpurea* glycoside from labeled sodium acetate in hydroponic culture. A modified chromatographic method was employed for the isolation of the glycoside fractions. A major fraction, approximately 0.13 per cent yield, of amorphous glycoside was obtained, and this was found to be a glycoside of the gitoxin group. The chemical properties and radioactivity measurement of this glycoside fraction and its aglycone are reported.

A PREVIOUS report from these laboratories (1) has described the accumulation and distribution of carbon^{14} in various tissues of *Digitalis purpurea* from carboxyl labeled sodium acetate added to plants in hydroponic culture. In view of the significant quantities of radioactive carbon found in root and leaf tissues of digitalis under the conditions described, it was deemed of interest to investigate the carbon^{14} distribution from the same source in the glycoside components. The fact that carbon^{14} from another source (carbon dioxide) can be utilized during digitalis glycoside biosynthesis has already been well established by Geiling, *et al.* (2). Further, there is much evidence reported in the literature (3–7) to indicate that an acetate pool plays an important role in the biosynthesis of other plant and animal steroid compounds. A relationship between acetate carbon and steroid glycoside biosynthesis in the case of the digitalis leaf glycosides has, however, not been reported.

EXPERIMENTAL

Using the technique of hydroponic culture for digitalis plants previously described by Djao and Youngken (1) plants of very young growth were placed into 1½-liter culture bottles (one plant per bottle) and grown under greenhouse conditions. A number of plants were similarly arranged for control purposes. Hoagland's solution, as modified by Djao and Youngken (1), was employed as the growth substrate. In order to assess the results of such acetate carbon^{14} feedings at two different periods, eleven plants were treated as a group during the first experiment and twenty plants were employed as a group for a repeat experiment several weeks later. Each plant was fed a single concentration of 100 μc. of carbon^{14} in 10 mg. of sodium acetate per 1 L. of culture solution at the period of one hundred and ten to one hundred and twenty days of growth. This represented a total moiety of about 0.00012 *M* sodium acetate per feeding. A ten-day period was utilized as most convenient for growth since during this time it had been observed previously (1) that maximum acetate-carbon activity (ca. 2%) could be obtained in the leaves and significant (ca. 4%) activity could be recovered in the roots.

At the completion of the ten-day growth period, leaves and roots were harvested, dried at about 29–38°, and powdered for glycoside isolation. Ap-

* Received August 27, 1954, from the College of Pharmacy, University of Washington, Seattle.

Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.

This study has been supported in part by the Washington State Funds for Medical and Biological Research (Initiative 171). Acknowledgment is also made to the Sandoz Co., Basel, Switzerland, and Department of Pharmaceutical Chemistry, University of Minnesota, for the gitoxin, and to Eli Lilly Co., Indianapolis, for the digitoxin employed.

An abstract of Part II of a dissertation submitted by E. H. Djao in partial fulfillment of the requirements for the Doctor of Philosophy degree, 1954, in the Graduate School of the University of Washington, Seattle.

proximately 5 Gm. per plant of dried leaf material was obtained, whereas less than 1 Gm. per plant of dried root material could be procured. It was felt that the small amounts of total root material would be inadequate for suitable glycoside analysis and this phase of the study was therefore not continued.

The separation of glycoside fractions from the leaf material was carried out as follows after the method of Geiling, *et al.* (2), with some modification:

Thirty-gram samples of powdered (No. 60 mesh) digitalis leaf material were mixed with 500 ml. of 50% alcohol and each mixture shaken overnight in an electric shaking machine. The mixture was then centrifuged, and the residue washed twice with 250-ml. portions of 50% alcohol. The combined solution and washings was evaporated to a thick paste at room temperature under a current of air. This was rinsed into a separatory funnel with about 15 ml. of water; 100 mg. of sodium bicarbonate was added, and the suspension was then extracted three times with 30-ml. portions of chloroform (U. S. P.) containing approximately 0.75% of alcohol. The chloroform extracts were then combined, dehydrated with desiccated sodium sulfate, filtered, and evaporated to dryness under reduced pressure. The residue was dissolved in 15 ml. of chloroform (reagent grade) containing 0.2% or less alcohol, and this solution was centrifuged to remove any insoluble particles and used for chromatographic adsorption.

Preliminary experiments with the use of digitoxin and gitoxin glycoside mixtures and nonradioactive digitalis leaf material indicated that chromatograms of alumina in addition to Decalso®[1] would remove most of the impurities to yield better isolation products. Therefore the method was modified to include this step. Alumina (80–200 mesh), which had previously been activated by heating at 150° for five hours, was packed to form 1 x 12-cm. columns. The columns were first washed with 30 ml. of chloroform (reagent grade), and the chloroform extract was then passed through it. The chromatogram was developed with 60 ml. of chloroform (U. S. P.). The eluates in each case were collected in 5-ml. portions and tested by the Keller-Kiliani test (8) for the presence of digitalis glycoside. Negative tests were obtained. The column was then eluted with chloroform containing 5.75% of alcohol. Positive Keller-Kiliani tests were obtained in this eluate, indicating the presence of digitalis glycosides. After passing about 60 ml. of the solvent through the column, the eluate portions ceased to give a positive glycoside test. This eluate was called the "crude glycoside fraction A." The column was then further eluted with a solvent of equal volumes of 95% alcohol and U. S. P. chloroform. Positive Keller-Kiliani tests were again obtained in this eluate, until about 60 ml. of the solvent had passed through the column and the eluate portions ceased to give a positive glycoside test. This eluate was called the "crude glycoside fraction B." Further elution of the column with 95% alcohol failed to produce any additional glycoside as indicated by the Keller-Kiliani test.

The crude fractions A and B were then separately purified by repeated chromatography using Decalso® adsorption columns similar to the procedure of Geiling.

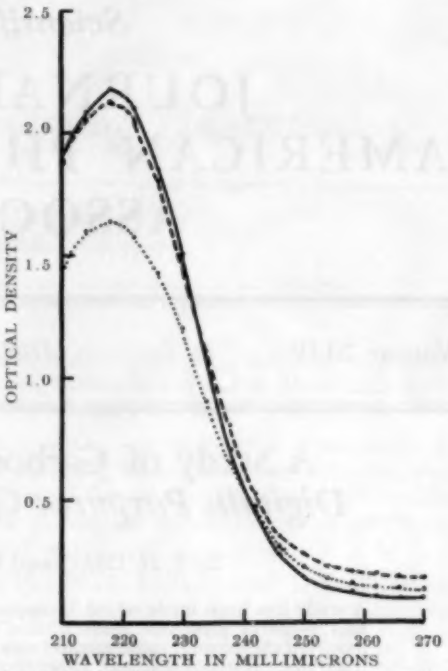

Fig. 1.—Ultraviolet absorption spectra for radioactive digitalis glycosides.

——— digitoxin
– – – – – glycoside fraction A
..... glycoside fraction B

Purification of the "Crude Glycoside Fraction A."—The "crude glycoside fraction A" obtained above was evaporated to dryness under reduced pressure. The residue thus prepared was dissolved in 10 ml. of chloroform (reagent grade), and filtered through an 8 x 80-mm. adsorption column of water-alcohol-chloroform washed Decalso® (80–100 mesh). The chromatogram was developed with 30 ml. of a solvent made by mixing 1 volume of 95% alcohol and 99 volumes of U. S. P. chloroform. The glycoside was eluted with 30 ml. of a solvent made by mixing 3 volumes of 95% alcohol and 97 volumes of U. S. P. chloroform. The eluate containing glycoside was next evaporated to dryness under reduced pressure and the residue dissolved in 10 ml. of chloroform (reagent grade). This solution was then readsorbed and eluted again from a fresh Decalso® column. The eluate containing glycoside was decolorized with Norite,[2] filtered, and evaporated to dryness under reduced pressure. The residue was then dissolved in 1 ml. of chloroform (reagent grade), and 9 ml. of petroleum ether (b. p. 40–60°) was added to precipitate the glycoside. The precipitate was centrifuged to remove the solvent, washed with 10 ml. of petroleum ether, and dried under reduced pressure. The product was a white

[1] Decalso obtained from the Permutit Company, New York.

[2] Norite obtained from R. W. Greef Co., New York.

powder. Approximately 40 mg. of powder (0.13%) was produced from 30 Gm. of powdered digitalis leaf material. This product was called the "amorphous glycoside fraction A."

Purification of the "Crude Glycoside Fraction B."—The purification of this fraction was done in the same manner as for the fraction A. The resulting product was a yellow-white powder. Approximately 5 mg. (0.017%) was produced from 30 Gm. of powdered digitalis leaf material, and this product was called the "amorphous glycoside fraction B."

Extensive efforts were made to purify further the glycoside fractions A and B above by the use of dilute alcohol, solvent mixtures of ether, acetone, and chloroform, and repeated adsorptions on alumina columns in an attempt to obtain crystalline glycoside products. These attempts were not successful. However, the quality of the white or pale yellow amorphous glycoside material which was separated (glycoside fractions A and B, respectively) was considered adequate for identification and radioactivity measurements. The yields of the leaf material and isolated glycoside fractions of the two experimental groups are tabulated in Table I.

sample per ml. of 95% alcohol in 1-cm. silica cells were used, and ultraviolet absorption spectra were made with a Cary Recording Spectrophotometer and hydrogen lamp. Results are shown in Fig. 1. The spectrum of the glycoside fraction A was almost identical with that of the digitoxin. Spectra of pure gitoxin samples were not made, because the pure crystalline gitoxin samples available for comparisons were very sparingly soluble in any solvent tried suitable for this purpose. However, since the same unsaturated lactone ring was responsible for the absorption spectrum, gitoxin could be expected to show a spectrum similar to that of digitoxin.

The quantity of glycoside fraction B obtained was considered too small and not sufficiently pure for further hydrolysis studies. Therefore, attempts were made to hydrolyze only the glycoside fraction A, in order to identify and measure the carbon^{14} activity of its aglycone.

Hydrolysis of Glycoside Fraction A.—The acid hydrolysis procedure of Smith (14) and Stoll and Kreis (15) for the purpurea glycosides was used for this purpose with some modification in order to adapt the method to small sample size. Fifty-milligram aliquots of glycoside were refluxed with

TABLE I.—DRY WEIGHT YIELDS OF DIGITALIS LEAF MATERIAL AND ISOLATED GLYCOSIDE FRACTIONS FROM HYDROPONIC CULTURE WITH CARBON^{14} CARBOXYL LABELED SODIUM ACETATE[a]

Number of plants	Experiment 1 — 11		Experiment 2 — 20	
	Dry Wt., Gm.	Per cent	Dry Wt., Gm.	Per cent
Total leaves	71	...	95	...
Total extraction material	60	...	90	...
Resultant glycoside fraction A	0.075	0.125	0.127	0.14
Resultant glycoside fraction B	0.0088	0.015	0.014	0.016

[a] Radioactivity supplied per plant: 1.25 x 10^8 c. p. m.

Characterization of the Glycoside Fractions.—The isolated amorphous glycoside fractions A and B were identified as follows: Each fraction was soluble in chloroform, acetone, 95% alcohol, and insoluble in water, ether, and petroleum ether. Positive reactions were obtained with Tollen's reagent[3] as indicated by the precipitation of silver. Positive reactions were also obtained when 0.5 mg. of each glycoside fraction was tested by the Legal test (9, 10) during which a deep red color formed. This substantiated the presence of unsaturation and a lactone ring. The glycoside fraction A gave a positive Keller-Kiliani reaction similar to that produced by a known sample of gitoxin during which a blue acetic acid layer and carmine-red intermediate zone were produced. This indicated the presence of glycosides of the gitoxin group. The amorphous glycoside fraction B gave a blue acetic acid layer and a red-brown color at the intermediate zone, suggesting a mixture of glycosides of both digitoxin and gitoxin groups or the presence of impurities.

The digitalis aglycones contain an α,β-unsaturated lactone ring, which normally shows absorption maxima close to 220 mμ (12, 13). Ultraviolet spectra studies for each of the above fractions yielded absorption similar to that of known glycoside samples of digitoxin. Solutions of 0.1 mg

[3] Tollen's reagent was prepared according to the method of Shriner and Fuson (11).

a mixture of 5 ml. of alcohol, 1 ml. of water, and 3 ml. of a 0.142 *N* hydrochloric acid, on a steam bath. After a period of thirty to sixty minutes, the mixture was cooled, neutralized with freshly prepared aqueous calcium hydroxide solution, and concentrated to approximately 3 ml. under reduced pressure, whereupon the aglycone was precipitated.

Preliminary experiments with 50-mg. aliquots of a pure gitoxin sample yielded approximately 15 mg. of aglycone, which readily crystallized in dilute alcohol, and had a melting point at 226° (uncorr.). The theoretical yield of gitoxigenin from gitoxin was 43.6%, or 21.8 mg. per 50 mg., and a reported melting point of 232° (corr.). Hydrolysis of a 50-mg. aliquot of the glycoside fraction A by the same procedure yielded approximately 15 mg. of a white powder. Attempts to crystallize it were unsuccessful. It melted slowly between 150–160°. The Keller-Kiliani test gave a carmine-red color at the intermediate zone indicating gitoxigenin, whereas no blue color was developed in the acetic acid layer, indicating the absence of digitoxose.

Radioactivity Measurements.—Radioactivity measurements were made by the methods previously reported (1) for acetate carbon^{14} labeled digitalis leaves, during which 50-mg. aliquots of powdered leaf material were oxidized by a wet oxidation procedure. The barium carbonate samples collected were washed with alcohol, plated on copper disks, and counted with the use of a Flow Counter

(Tracerlab). Aliquots of 5 mg. each were employed in the same manner for measuring the radioactivity of fraction A and B glycosides and the aglycone obtained from fraction A. Approximately 1% of the total carbon[14] supplied during the acetate-carbon[14] feedings was recovered in the leaf material of each group. This agrees with the findings previously reported (1) for the per cent recovery of carbon[14] in digitalis leaf from an acetate-carbon source. The results of radioactivity measurements of the leaves, glycoside, and aglycone fractions of the material from both experimental groups are shown in Table II.

The specific activity of counts per minute per mg. of carbon found in the total leaf materials (528 and 947) was much higher than those found for the glycoside fractions of the leaf (305 and 262). This indicated that certain other substances of higher specific activity were present in the leaf material. Krotkov and Barker (16), who studied the utilization of acetate-carbon in tobacco leaves, reported that the acetate-carbon was largely converted into water-soluble acids and then rapidly respired. Christensen and Wang (17) similarly studied the utilization of acetate-carbon in tomato plants. Their results indicated that large amounts of the

TABLE II.—RADIOACTIVITY MEASUREMENTS OF DIGITALIS LEAF, GLYCOSIDE AND AGLYCONE FRACTIONS AFTER A TEN-DAY FEEDING WITH CARBON[14] CARBOXYL LABELED SODIUM ACETATE[a]

	Experiment 1		Experiment 2	
Number of plants	11		20	
	c. p. m./mg.	Per cent	c. p. m./mg.	Per cent
Dried leaves:				
Carbon content[b]	...	33.8	...	33.9
Specific activity of carbon	528	...	947	...
Specific activity of sample	179	...	320	...
Calculated total carbon[14] recovery	...	0.92	...	1.22
Glycoside fraction A:				
Carbon content[b]	...	60.9	...	64.2
Specific activity of carbon	305	...	262	...
Specific activity of sample	186	...	168	...
Calculated total carbon[14] recovery;				
from total acetate supplied	...	0.0011	...	0.0009
from plant leaf material	...	0.118	...	0.074
Glycoside fraction B:				
Carbon content[b]	...	60.0	...	60.5
Specific activity of carbon	305	...	254	...
Specific activity of sample	183	...	154	...
Calculated total carbon[14] recovery;				
from total acetate supplied	...	0.00014	...	0.000092
from plant leaf material	...	0.015	...	0.0075
Aglycone fraction A:				
Carbon content[b]	...	66.0	...	...
Specific activity of carbon	265	...	...	...
Specific activity of sample	175	...	...	...

[a] Radioactivity supplied per plant: 1.25×10^9 c. p. m.
[b] Carbon content calculated from barium carbonate obtained by wet oxidation.

DISCUSSION

From these results it is apparent that carbon[14] from the labeled sodium acetate was incorporated in the two glycoside and one aglycone fractions, but in very small amounts. On the basis of the Keller-Kiliani test and other identification studies, the highly concentrated glycoside fraction obtained was probably of the gitoxin type. There is evidence, therefore, that carbon[14] from the exogenous acetate could have been used in the biosynthesis of this glycoside. Difficulties encountered in the crystallization of the aglycone portion of the fraction prevented a definite characterization of the genin and determination of the carbon[14] in it.

If under the conditions of the experiment the aglycone of fraction A had been completely purified and found to contain all of the carbon[14] of the fraction, the rate of incorporation of radioactive carbon[14] would still have been rather low. By calculation approximately equal activity would then be established in the carbon of the sugar portion of the glycoside.

acetate-carbon were respired and that the remaining carbon was found mostly in water-soluble acids such as citric and malic acids. In the previous report (1) it was shown that 5.95% of the total carbon[14] supplied was recovered as carbon[14] dioxide during a twelve-hour dark period, and that 3.2% of the same was released from the area of the culture solution. This indicated that approximately 2.75% of the total carbon[14] supplied had been respired by the leaves during a twelve-hour dark period, whereas the highest carbon[14] recovery found in dried leaf material was only 1.81% in ten days. Therefore it can be concluded that the carbon[14] in digitalis plants from the acetate source was mostly respired, and that the carbon[14] remaining in digitalis leaf material might have been confined mostly to certain respiration substrates, e. g., water-soluble acids, as has been indicated to be the case in other plants. The study of the acetate-carbon[14] in components other than glycosides is being continued.

It must then be concluded that, under the experimental conditions herein reported, the pathway of acetate-carboxyl-carbon to glycoside was exceedingly indirect.

SUMMARY

A study has been made of the role of certain acetate feedings in the glycoside formation of *Digitalis purpurea* with the use of hydroponic culture methods and isotope tracer techniques.

Approximately 0.13 per cent total amorphous glycosides were isolated from leaf material in a highly concentrated form. This was considered to be largely glycoside of the gitoxin type. Radioactive carbon[14] from the acetate source was present in the glycoside fractions but in very small quantities.

An aglycone fraction was separated from the gitoxin glycoside fraction and found to contain carbon[14]. However, the specific radioactivity of carbon in this aglycone fraction was significantly small.

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Some Pharmacological Properties of Phenyl *alpha*-Naphthyl Ketimine*

By CECIL P. HEADLEE and JACK H. KEITH

Phenyl *alpha*-naphthyl ketimine hydrochloride has been prepared and studied for anticonvulsant and certain other pharmacological properties. Definite anticonvulsant activity was shown against both Metrazol® and electric shock-induced convulsions. A positive local anesthetic, hypotensive and antipyretic effect was observed but analgesic, autonomic, and irritant activities were essentially negative. Symptoms of toxicity did not occur with either single doses or with prolonged administration.

DURING the past several months many ketimines have been subjected to a preliminary pharmacological investigation, and some of the compounds tested have been found to be effective in suppressing Metrazol®-induced convulsions (1).

On the basis of these laboratory observations, and because of the lack of pharmacological information regarding phenyl *alpha*-naphthyl ketimine, first prepared by Migonac in 1920 (2), this compound has been prepared as the hydrochloride using the method of Pickard and Vaughan (3) and subjected to a pharmacological study.

EXPERIMENTAL

Determination of Anticonvulsant Activity.—*Anti-Metrazol Effect.*—In evaluating phenyl α-naphthyl ketimine hydrochloride for anti-pentamethylenetetrazol (Metrazol®)[1] activity, five Wistar-strain eighteen-hour fasted rats of mixed sex and weighing between 100 and 150 Gm. were used for each dose level. The material, too insoluble in water to be administered as an aqueous solution, was prepared as a 10% suspension in 5% tragacanth mucilage and administered perorally in doses of 65, 125, 250, 500, and 1,000 mg./Kg. Employing the method of Chen, Portman, Ensor, and Bratton (4) (the technique used in the ketimine study previously mentioned) and after waiting from thirty to forty-five minutes for absorption of the test drug, the animals were injected subcutaneously with an aqueous solution of Metrazol® in the amount of 93 mg./Kg. The animals were then observed thirty-six minutes for convulsive seizures. The severity of the convulsive seizures, the criterion of the test, was less pronounced at the 125 mg./Kg. dose level, and complete protection against Metrazol® was afforded at 1,000 mg./Kg.

In a second similar test using a group of 10 animals and a 3% suspension of the ketimine in 5% tragacanth mucilage, it was observed that 125 mg./Kg. completely prohibited Metrazol®-induced convulsions for a period of two hours, when injected intraperitoneally.

Effect on Increasing the Abnormally Lowered Convulsive Threshold to Metrazol.—Using the method of Swinyard, Toman, and Goodman (5) of lowering the convulsive threshold of the rat to Metrazol®, 15 adult Wistar-strain eighteen-hour

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[1] Generously supplied by the Bilhuber-Knoll Corporation.

fasted rats were orally hydrated with water at a dosage of 5 cc./100 Gm., every thirty minutes for four doses. Ten of these animals were used for testing purposes and received 125 mg./Kg. of the ketimine in the last dose of water. Thirty minutes after administration of the test drug, these animals were injected subcutaneously with 60 mg./Kg. of a 3% aqueous solution of Metrazol®. Two of the animals exhibited convulsions while the remaining eight animals showed only a slight hyperexcitability. All animals in the control group, receiving a subcutaneous dose of only 35 mg./Kg. of the solution of Metrazol® exhibited convulsive seizures. These results clearly indicate that the ketimine elevated the Metrazol® seizure threshold.

Effect Against Electroshock Seizures.—Electroshock apparatus as patterned after the suggestions of Putnam and Merritt (6) was used in studying the anticonvulsant effectiveness of phenyl α-naphthyl ketimine against electroshock convulsions. A group of female Wistar-strain rats, weighing approximately 100 Gm. was selected for the experiment. The occipital electrode used consisted of a sponge wet with saline solution, and the other electrode was an iron bit made so that it could be inserted into the mouth of the animal. While receiving the electroshock, the animals were restrained by a stockade. Before beginning the test proper, it was necessary first to establish a procedure which would insure that each rat would receive a convulsive shock. Consequently, all animals were tested, at twenty-four-hour intervals, to establish this level. Each animal was placed in the stockade and the head and mouth electrodes were attached. These electrodes were connected to the output of a varitan-type transformer which allowed the experimenter to vary the voltage applied to the electrodes. An alternating current milliammeter was connected in series with this circuit. The voltage was then adjusted until the animal exhibited electroshock convulsions. From the original group of animals, three groups of eight rats were selected which exhibited electroshock seizures when the current indicated by the milliammeter was 6 ma. The voltage was varied in each experimental session until the 6-ma. level was obtained. This level was maintained for fifteen seconds. Perorally, group 1 received 20 mg./Kg. of a 0.4% aqueous solution of sodium phenobarbital, group 2, 100 mg./Kg. of a 2% suspension of the ketimine in 5% tragacanth mucilage and group 3 received nothing and served as a control. Forty minutes after administration of the drugs, the animals were subjected to the electroshock. The criterion of the test was the complete abolishment of convulsive seizures. This test was carried out three times at a frequency of once every twenty-four hours, and the groups were rotated so that no group received the same treatment twice. As determined from three testings, the mean protection afforded by phenobarbital was 63% and the ketimine, 58%.

In a second study, two groups of five Wistar-strain rats of mixed sex, weighing 125–220 Gm. were utilized. When shocked at 7 ma. for ten seconds, forty minutes after oral administration of 20 mg./Kg. of sodium phenobarbital to one group and 250 mg./Kg. of the ketimine to a second group, the former showed a protection of 70% and the latter, a protection of 55%.

FURTHER PHARMACOLOGICAL EVALUATIONS

Acute Toxicity.—When a 10% suspension of phenyl α-naphthyl ketimine in 5% tragacanth mucilage was administered orally to five adult Wistar-strain rats of mixed sex, the only gross observable symptom at a dose of 500 mg./Kg. was a slight hypnosis. Using a 20% suspension of the ketimine in 5% tragacanth mucilage, it was further observed that higher doses produced increasing hypnotic effects and complete narcosis was reached when a dose of 5,000 mg./Kg. was administered. Two animals were used at each dose level and in no instance was death produced. These results indicated that death would be produced only at a very high dose level and due to the limited quantity of the test drug, a determination of the LD_{50} was not feasible.

Local Effects.—No symptoms of irritation were exhibited when a 1% ointment of phenyl α-naphthyl ketimine in petrolatum was rubbed into the shaven and nicked skin of six guinea pigs. Further, when doses of 1 to 4 mg. of the test drug, suspended in 0.2 cc. of equal parts 1% saline and propylene glycol were injected intracutaneously into the backs of six guinea pigs, there was little evidence of irritation. A slight induration and yellow discoloration were the only observable reactions. Pricking the areas of injection with a pin elicited no response for a period of fourteen hours, thereby indicating a local anesthetic effect. A group of six control animals exhibited no symptoms of irritation or local anesthesia when injected intracutaneously with 0.2 cc. of a 50% solution of propylene glycol in 1% saline.

Chronic Toxicity.—A group of 15 Wistar-strain rats of mixed sex weighing 125–220 Gm. were selected for this test. Ten of the animals were administered perorally a 5% suspension of the ketimine hydrochloride in 5% tragacanth mucilage at a dose level of 250 mg./Kg. daily for thirty days. The remaining five animals served as controls and were administered 5% tragacanth mucilage, orally, and in the same proportionate volume as the test animals. The animals were examined daily and other than a decrease of 60–80% in the urinary volume during the first five hours following administration of the ketimine, nothing unusual was noted. The urine was examined weekly and in all cases, Benedict's test for blood sugar, and Lange's and Gerhardt's tests (7) were negative. Negative results were also obtained from Smith's, Gmelin's, and Hays' test (7) for bile, Ehrlich's test (7) for urobilinogen and urobilin, the benzidine test for hemoglobin, and Watson's and Swartz's test (7) for porphobilinogen. Color tests gave no evidence of increase in allantoin excretion. The animals were sacrificed at the end of thirty days and autopsies revealed no gross pathological changes.

Tissue sections of the heart, kidney, intestine, liver, lung, testes, adrenal gland, and spleen were prepared. A microscopic pathological examination of these stained sections did not reveal any significant changes to have taken place with a prolonged administration of the test drug.

Uretic Effect.—In view of the uretic effects of phenyl α-naphthyl ketimine observed during the chronic toxicity study, 37 rats, Wistar-strain of mixed sex and weighing 72–258 Gm. were utilized

to study further the effect of the test drug on urine flow as produced by single doses. A normal urinary output for a five-hour and an eighteen-hour period was established through the use of metabolism cages in the usual manner. Food was withdrawn eighteen hours prior to the test and normal saline, 25 cc./Kg. containing the same amount of 5% tragacanth mucilage as the animals received later in the test proper, was administered perorally. Three days later the rats were administered peroral doses of a 3% suspension of the ketimine in 5% tragacanth mucilage, in sufficient amount of saline to bring the total volume to 25 cc./Kg. Fifteen of the animals received doses of the ketimine in the amount of 125 mg./Kg. while the remaining 22 received 250 mg./Kg. When a comparison was made of the urinary output between the normal and treated animals, no influence on the urinary volume was noted.

Blood Pressure Effect.—The blood pressure effect of phenyl α-naphthyl ketimine hydrochloride was studied utilizing the photoelectric tensometer and the method of Kersten, Brosene, Ablondi, and SubbaRow (8) for the indirect determination of blood pressure changes in the rat. Twelve Wistar-strain rats of mixed sex, weighing 160–275 Gm. were placed repeatedly in the rat holder of the photoelectric tensometer until the animals had become familiar with the holder and the manipulations necessary in using the machine. Of this selection of 12 animals, four were administered 250 mg./Kg. of a 5% suspension of the ketimine in 5% tragacanth mucilage, 4 were administered 500 mg./Kg. of a 10% suspension of the ketimine in 5% tragacanth mucilage and four were administered 30 mg./Kg. of a 1% aqueous solution of sodium nitrite. The drugs were administered orally and thirty minutes was allowed for absorption of the ketimine and twenty minutes for absorption of the sodium nitrite before blood pressure determinations were made. From four blood pressure readings, taken over a period of twenty minutes, was calculated the average blood pressure determination. Although the ketimine did show a hypotensive effect at both levels (a 17% and 15% decrease, respectively, for the two dose levels administered), it was inferior to the hypotensive effect of sodium nitrite (18% decrease) both from the standpoint of degree of action and duration of action.

Autonomic Effects.—Phenyl α-naphthyl ketimine showed no significant autonomic effects when (*a*) tested on isolated rabbit ileum suspended in Tyrodes' solution, (*b*) tested *in vivo* in rats against Mecholyl® or (*c*) tested *in vivo* in mice against the effects of epinephrine.

Analgesic Effect.—Because previous studies indicated possible analgesia, further consideration was given to this type of action. The radiant heat method (9) was utilized and healthy rats of either sex Wistar-strain, weighing 125–175 Gm. were used as test animals. An 8% suspension of the ketimine hydrochloride in 5% tragacanth mucilage was administered orally to seven rats at a dose level of 100 mg./Kg., to five rats at a dose level of 250 mg./Kg. and to five rats in the amount of 500 mg./Kg. The animals were tested for an increased reaction time beginning thirty minutes after drug administration. These tests were made at fifteen-minute intervals over a period of one hour and at no time was there observed any increase over the normal reaction time. When a 2% suspension of the test drug in 5% tragacanth mucilage was injected intraperitoneally into four groups of three rats each at doses of 20, 40, 65, and 85 mg./Kg., and when these animals were observed for analgesia in the same manner as with the oral doses, again no increased reaction time was noted.

Antipyretic Effect.—Nine adult white rabbits divided into three groups of three each were selected for this phase of investigation. One group served as a control while the other two groups were used to test for antipyretic effects of acetanilid and the ketimine. After obtaining an average normal rectal temperature for each animal, the standard, acetanilid, 200 mg./Kg., and the test drug, 200 mg./Kg., both prepared as 10% suspensions in 5% tragacanth mucilage, were administered orally to two groups of three animals respectively. Four cc. of tap water, to produce fever, was then injected into a lateral ear vein of the six test animals, and also into the remaining three animals which served as controls. Rectal temperature readings were then recorded each hour for three hours and the net reduction of temperature for each group was calculated. Acetanilid was observed to produce a net temperature lowering of 55% from that of the controls, while the ketimine produced only a 38% reduction.

CONCLUSIONS

1. Although effective in increasing the convulsive threshold to Metrazol® in the hydrated rat and although antagonistic to electroshock and Metrazol®-induced convulsive seizures, in general it may be said that phenyl *alpha*-naphthyl ketimine hydrochloride is inferior to phenobarbital sodium as an anticonvulsant, when a dose versus response comparison is made.

2. With regard to untoward effects such as irritation, acute and chronic toxicity, evidence has been presented that the test drug is of a low toxicity in rats.

3. Local anesthetic, antipyretic, and hypotensive effects are demonstrable.

4. No significant autonomic, uretic, or analgesic effects were observed.

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The Synthesis of *para*-Aminosalicylic Acid*

I. The Synthesis of C-14 Carboxyl-Labeled *p*-Aminosalicylic Acid

By LEON CLARK, ALFRED HELLER†, and LLOYD J. ROTH

A procedure is described for the synthesis of C-14 carboxyl-labeled *p*-aminosalicylic (4-amino-2-hydroxybenzoic) acid. The Sandmeyer reaction, using potassium radiocyanide, is employed in the synthesis of *p*-nitrosalicylic acid, which is, in turn, reduced by catalytic hydrogenation at room temperature.

PARA-AMINOSALICYCLIC ACID (PAS), as a result of its widespread use in the therapy of tuberculosis, has been the subject of considerable medical and pharmaceutical research. When, in the course of such investigation in this laboratory, the chemically pure compound isotopically labeled in the carboxyl group was required as a tool, no wholly suitable method for its preparation could be found among those previously described. For convenience, the existing syntheses can be divided into two categories: those in which *p*-nitrosalicylic acid is reduced (1, 2, 3), and those in which *m*-aminophenol is carboxylated (4, 5, 6). The former group affords nonambiguity, but, of existing procedures for the synthesis of *p*-nitrosalicylic acid, none permits the convenient incorporation of isotopic carbon into the carboxyl group. The latter, or Kolbe synthesis, is not free of ambiguity, since a number of structurally related by-products are possible. One of these, a di-carboxylated *m*-aminophenol, has been isolated from PAS which was prepared by this procedure (7, 8). The importance of an unambiguous synthetic route is strongly emphasized by the fact that the physical properties of this compound are of little value in establishing either the presence or the absence of possible by-products (8, 9). We were thus led to investigate the possibility of preparing *p*-nitrosalicylic acid via the Sandmeyer reaction, and reduction of this compound under conditions which would circumvent the tendency of the aminosalicylic acid to decarboxylate. The resultant synthetic scheme fulfills the specific need for an unequivocal route to the isolation of chemically pure PAS. Furthermore this procedure provides a method for the synthesis of C-14 carboxyl-labeled PAS.

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† Predoctoral Fellow, National Science Foundation.

EXPERIMENTAL[1]

5-Nitro-*o*-aminophenol (2-Hydroxy-4-nitroaniline).—ON-Diacetyl-*o*-aminophenol was prepared by acetylating *o*-aminophenol hydrochloride (10) (Eastman, practical). After extensive purification, this compound was nitrated, in accordance with the method of Ingold (11), to give a mixture of 5-nitro and 3-nitro-ON-diacetyl-*o*-aminophenol. A separation of these isomers was effected through a series of fractional recrystallizations from ethanol, augmented by aqueous flotation.[2] The crude 5-nitro derivative was obtained as a mass of downy needles. After five slow recrystallizations from ethanol, 5-nitro-ON-diacetyl-*o*-aminophenol was collected as a mat of filamental needles resembling glass wool; m. p. 267–269°, as reported by Ingold.

Alkaline hydrolysis (11) of this compound led to the isolation of 5-nitro-N-acetyl-*o*-aminophenol, which, after purification, was obtained as small yellow prisms; m.p. 267–269° (decompn), in agreement with the reported value.

The N-acetyl compound (8 Gm.) was refluxed for three hours with a mixture of 25 ml. of ethanol and 25 ml. of concentrated hydrochloric acid. The resulting solution was taken to dryness *in vacuo*. The residue was taken up in boiling water and neutralized by treatment with sodium acetate. After cooling the mixture to 0°, the precipitate was collected and recrystallized from water. In this manner, 5-nitro-*o*-aminophenol was obtained as golden brown crystals; m. p. 200–202° (decompn.), as compared with the reported (10) values of 201–202° (decompn.). Yield: 4.39 Gm. (70%).

2-Hydroxy-4-nitrobenzonitrile.—A procedure reported for the synthesis of 2,4-dinitrobenzonitrile (12) proved extremely valuable in our effort to find the rather limited set of conditions under which the Sandmeyer reaction can be employed in the production of this nitrile. A highly efficient stirring procedure is indispensable, in view of the tendency of the intermediate phenolic diazonium compound to couple, with a resultant yield of tars. Accordingly, a Waring Blendor, operated at 30 volts, was used as a reaction vessel.

5-Nitro-*o*-aminophenol (1.75 Gm., 11.3 mM) was dissolved in 20 ml. of warm glacial acetic acid and diazotized according to the method of Hodgson and Walker (13).

[1] All melting points are uncorrected. Microchemical analyses were performed by William Saschek, of the Department of Chemistry, University of Chicago.

[2] The desired 5-nitro derivative tends to float when stirred with water, hence can be decanted.

In the meantime, a mixture of potassium cyanide (3.25 Gm., 50 mM), nickel sulfate, $NiSO_4 \cdot 6H_2O$ (2.88 Gm., 11 mM), sodium carbonate (20 Gm. anhydrous), and water (20 ml.) was prepared in the jar of the Waring Blendor. Stirring was started, and maintained throughout the operation.

The filtered solution of the diazonium sulfate was added at such a rate that the temperature did not exceed 30°. This addition, which required about two hours, was interrupted at intervals to permit the addition of more sodium carbonate, in order to keep the *p*H within the weakly alkaline range. When all the diazonium solution had been added, the reaction mixture was transferred to a round-bottom flask and heated at 90°, for thirty minutes, with vigorous stirring. The mixture was then cooled in an ice bath, and the *p*H was adjusted to 6.0–6.5, whereupon the crude nitrile precipitated out as fine crystals. Purification by sublimation *in vacuo* gave the nitrile as a yellow powder; m. p. 160–161°, in agreement with the reported value for 2-hydroxy-4-nitrobenzonitrile (14). Chemical yields, based upon 5-nitro-*o*-aminophenol, ranged from 43 to 65%.

Since, ultimately, the cyanide would be the labeled component, attempts were made to lower the ratio of cyanide to aminophenol. Such attempts invariably resulted in the increased formation of tars and a lowering of chemical yield.

***p*-Nitrosalicylic Acid.**—Hydrolysis of this nitrile, either by phosphoric acid (15) or by sulfuric acid (3), gave *p*-nitrosalicylic acid, which, after purification, was obtained as white, lustrous needles; m. p. 234–235°. Reported m. p. 234–235° (3).

***p*-Aminosalicylic Acid.**—*p*-Nitrosalicylic acid (598 mg., 3.27 mM) was dissolved in absolute ethanol (50 ml.). Platinum oxide catalyst (50 mg.) was added and the mixture was hydrogenated at room temperature and a pressure of 1 atmosphere until hydrogen uptake ceased. At this point the theoretical amount had been absorbed. The operation was continued for an additional thirty minutes, then the catalyst was removed by filtration.[3] The solvent was removed *in vacuo*, and the residue was dissolved in the mininum amount of absolute methanol. This solution was treated with charcoal, at room temperature, and filtered. Upon the addition of about five volumes of ice-cold water to this colorless filtrate, *p*-aminosalicylic acid crystallized out as fine, snow-white needles; m. p. 147–148°, with effervescence. Yield: 0.400 Gm. (62%). Reported m. p. 147° (9).

Anal.—Calcd. for $C_7H_7NO_3$: C, 54.90; H, 4.57; N, 9.15. Found: C, 54.99; H, 4.79; N, 9.27.

The melting point of our product was determined by the capillary method, using an oil bath, preheated to 145°, and a temperature increase of 3° per minute.

The compound was found to be soluble in water to the extent of 0.1% w/v, soluble in ether to the extent of 1.2% w/v, and moderately soluble in alcohol.

The ultraviolet absorption spectrum in alcoholic solution proved identical with that reported by Stelt (8).

A sample of this compound (0.100 Gm.) was converted via the Sandmeyer reaction to the corresponding chlorosalicylic acid. The melting point of this derivative, 207–209°, was in agreement with that reported for 4-chlorosalicylic acid (16), (206–207°; 211–212°). Of the remaining isomers (16), none melts above 180°.

Through use of this procedure, with the potassium cyanide replaced by potassium radiocyanide, we have synthesized carboxyl-labeled *p*-aminosalicylic acid as a chemically pure compound. In agreement with theory, the compound thus obtained showed one-seventh the specific activity of the radiocyanide used. Radiochemical purity was verified by paper chromatography.

The 4-chlorosalicylic acid derivative was prepared and found, after repeated recrystallization, to have the same specific activity as the C-14 labeled PAS.

SUMMARY

1. The physical properties of *p*-aminosalicylic acid because of their empirical nature, are not, within themselves, sufficient to establish chemical purity of the compound.

2. Thus, where chemical purity is vital, as in the employment of isotopic tracer techniques, the establishment of such purity must depend largely upon an unequivocal synthetic route and the agreement of microchemical data.

3. The Sandmeyer synthesis of 2-hydroxy-4-nitrobenzonitrile is described.

4. This procedure makes possible the convenient preparation of the carboxyl-labeled compound from potassium radiocyanide.

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[3] Solutions of *p*-aminosalicylic acid, in contact with active catalyst, darken rapidly on exposure to air; therefore, filtration was carried out under nitrogen, using an ultrafine sintered-glass filter.

Isolation of an Alkaloid from *Vinca Minor**

By STANLEY SCHEINDLIN and NATHAN RUBIN

The literature concerning the genus *Vinca* has been reviewed. Extraction of *Vinca minor* L. (Fam. *Apocynaceae*) with ammonia and benzene yielded a crude alkaloidal fraction. From the crude alkaloid, by a chromatographic process, a crystalline alkaloid has been isolated. The empirical formula, molecular weight, melting point, ultraviolet and infrared spectra, and color reactions of this alkaloid are reported.

Vinca minor L. (Fam. *Apocynaceae*), the lesser periwinkle, is now considered merely an ornamental plant, useful as a ground-cover especially on steep banks and under trees. Yet this plant has a long, though undistinguished, history of medicinal use. It was mentioned as a drug by both Dioscorides and Galen (1), has enjoyed some use in European folk medicine (2), and is still official in the French Codex under the title of "Pervenche officinale" (3).

There has been no thorough study of the constituents of any of the species of the genus *Vinca*. However, the presence of alkaloids has been reported in several of these species, namely *V. lancea* (4), *V. herbacea* (5), *V. pubescens* (6, 10), *V. rosea* (7, 8), *V. major* (9), and *V. minor* (6). Several alkaloids have been isolated in crystalline form. These together with their physical constants are tabulated below:

V. pubescens: Orechoff, Gurewitch, and Norkina (10).

(*a*) *Vinine*, colorless needles, $C_{19}H_{28}N_2O_4$, m. p. 211–213°, $[\alpha]_D$ −70.12°, (c, 1.6 in absolute alcohol). Hydrochloride, m. p. 212° (decompn.); sulfate, m. p. 229–230° (decompn.); chloroplatinate, m. p. 226–227° (decompn.).

(*b*) *Pubescine*, yellow needles, $C_{20}H_{28}N_2O_4$, m. p. 227–228°, $[\alpha]_D$ −134.2°, (c, 0.298 in absolute alcohol).

(*c*) An unnamed alkaloid, in minute yield: colorless needles, m. p. 194–195°, which strongly depressed the melting point of vinine.

V. rosea: Paris and Moyse-Mignon (8).

(*a*) *Vinceine*, $C_{21}H_{26}N_2O_3$, m. p. 259–260°, $[\alpha]_D$ −56°, (c, 0.5 in chloroform), absorption maxima at 225 and 280 mμ, absorption minimum at 265 mμ. Hydrochloride, m. p. 290° (decompn).

V. major: Janot and LeMen (9).

(*a*) *Reserpinine* (11-methoxy-δ-yohimbine), colorless prisms, $C_{22}H_{28}N_2O_4$, m. p. 242°, $[\alpha]_D$ −109°, (c, 0.5 in pyridine), absorption maxima at 229 and 299 mμ, inflection at 250 mμ.

(*b*) Colorless needles, m. p. 316°, absorption maxima at 240 and 310 mμ. Possibly identical with *serpinine*.

Within the last few years, *Rauwolfia serpentina* and its alkaloids have come into great prominence in the treatment of hypertension. Concurrently, chemical research has shown that the basic ring structures of the alkaloids of various *Apocynaceae* such as *Rauwolfia*, *Iboga*, and *Alstonia*, are essentially similar. The importance of *Vinca minor* as a potential source of related hypotensive alkaloids thus becomes apparent.[1] *Vinca minor*, furthermore, grows in the United States, and can be easily cultivated if necessary, whereas the other drugs mentioned above must be imported from distant places all over the world.

EXPERIMENTAL

The leaves and stems of *Vinca minor* were collected in Jenkintown, Pennsylvania, during the months of August, September, and October. The plant material was washed, dried first in the air and then in an oven at 40°, and finally ground to a coarse powder. Before being ground, each batch was authenticated by Dr. Theodor P. Haas.[2]

Extraction of Crude Alkaloids.—The limitations imposed by the apparatus available made it most convenient to extract the drug in 200-Gm. batches.

Two hundred grams of *Vinca minor* was moistened with 130 cc. of 10% ammonia. The moist drug was packed in a Soxhlet apparatus, covered with benzene, and macerated for twenty-four hours or longer. The drug was then exhausted by continuous extraction. The benzene extract was evaporated to dryness in a current of air, no heat being applied The residue was extracted with several portions of 0.5% hydrochloric acid. The acid solutions were made alkaline with 28% ammonia. The precipitate which formed was allowed

* Received August 27, 1954, from the Department of Chemistry, Philadelphia College of Pharmacy and Science. Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.

[1] After the presentation of this paper before the Scientific Section, a personal communication from Dr. Jack Cooper of Ciba Pharmaceutical Products indicated that Schlittler and Furlenmeier [*Helv. Chim. Acta*, **36**, 2017(1953); *Chem. Abstr.*, **49**, 1006(1955)] had isolated from *Vinca minor* an alkaloid which they named *vincamine*. This alkaloid, $C_{21}H_{26}N_2O_3$, m. p. 232–233°, $[\alpha]_D^{23}$ +41°, shows the typical ultraviolet absorption maxima of an indole nucleus. Vincamine appears to be a different alkaloid from the one whose isolation is described in this paper. This conclusion is based on physical constants, elementary composition, and a color reaction mentioned by Schlittler and Furlenmeier. Vincamine gives no red color with nitric acid, whereas the alkaloid reported in this paper does.

[2] Department of Biology, Philadelphia College of Pharmacy and Science.

to stand one hour, then filtered with suction, washed with distilled water, and dried at room temperature or 40°. The crude alkaloidal fraction thus obtained had a faint pink color. The yields ranged from 0.4 to 0.5%, based on the dried drug.

Isolation of a Crystalline Alkaloid.—In a typical experiment, 0.7 Gm. of crude alkaloidal fraction was triturated with five 30-cc. portions of anhydrous ether. A red solution resulted, which was separated by filtration from some brown insoluble matter. The pooled ether extracts were passed through a column, 19 mm. in diameter, containing 10 Gm. of Brockmann alumina. After nearly all the ether had passed through, 20 cc. more anhydrous ether was added to the column. Two additional 20-cc. portions of ether were used in the same way. Passage through the column resulted in adsorption of the red color of the solution in a firmly-held band at the top, while the eluate was colorless or faintly yellow.

The solution which had passed through the column was allowed to evaporate spontaneously in a dark place. Rosettes of white needles, embedded in a yellowish oil, were obtained. Treatment with ice-cold absolute alcohol dissolved the oil, and the crystals were collected on a sintered-glass Buchner funnel, and washed with many portions of cold absolute alcohol. Yields of crystalline alkaloid ranged up to 7%, based on the crude alkaloidal fraction.

The oily material mentioned above is also alkaloidal, but it has thus far resisted all efforts to crystallize it.

Properties of the Crystalline Alkaloid.—The melting point of the white crystals, determined in a capillary using a total immersion thermometer, was 201–202° with decomposition. The melt was deep red. The melting point varied somewhat with the rate of heating of the bath.

Combustion analysis[3] indicated the following percentage composition:

Carbon	69.02	68.77
Hydrogen	6.62	6.63
Nitrogen	7.06	7.05
Oxygen (by difference)	17.30	17.55

Determination of the molecular weight by the Rast method (11) indicated a molecular weight of 386.

Based on the analytical and molecular weight data presented, the empirical formula of the alkaloid was computed to be $C_{23}H_{26}N_2O_4$.

Ultraviolet Absorption Spectra.—The ultraviolet absorption spectrum was determined using a Beckman Quartz Spectrophotometer—Model DU. Figure 1 shows the curve obtained at a concentration of 8.03 mg. per liter in 95% ethanol. The alkaloid exhibited absorption maxima at 229 and 274 mμ, and an inflection at 290 mμ. Its absorption minima were at 251 and 288 mμ.

The absorption spectrum in 0.05 *N* hydrochloric acid showed a hypsochromic shift, with maxima at 222 and 269.5 mμ respectively, an inflection at 289 mμ, and minima at 246.5 and 287 mμ, respectively.

The spectra obtained were similar to those given by alkaloids having an α,β-substituted indole nucleus (12, 13).

[3] Clark Microanalytical Laboratory, Urbana, Ill.

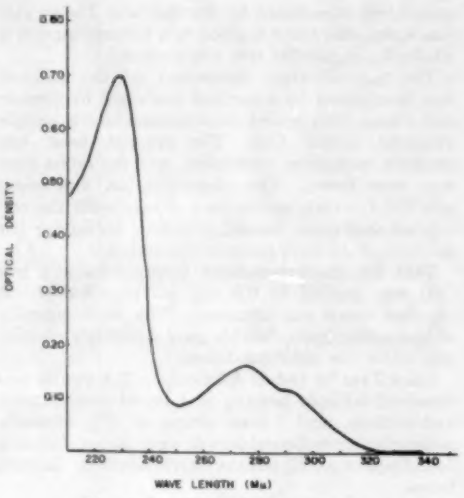

Figure 1.—Ultraviolet spectrum.

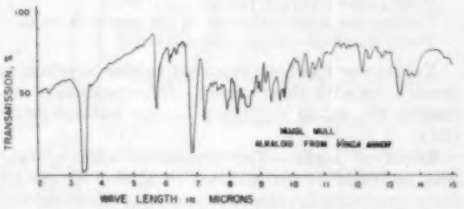

Figure 2.—Infrared spectrum.

Infrared Absorption Spectrum.[4]—The infrared absorption spectrum was determined in a Nujol mull, using a Perkin-Elmer Model 21 instrument. The curve obtained is shown in Fig. 2.

The strong bands at 3.4, 6.8, and 7.25 μ are due to the Nujol. No band was found around 3 μ. This would indicate the absence of the NH group. However, decarboxycorynantheine and dihydrocorynantheane, which are known to have an indole nucleus also fail to show the NH band in the infrared (12).

The strong band at 5.75μ is indicative of the ester group. The bands found between 6.15 and 6.70μ can be attributed to the benzenoid ring (14, 15) and have been stated to be typical of indoles (16). A strong band at 7.95μ may be attributable to a methoxyl group attached to an unsaturated carbon atom (12). A band at 9μ has been attributed to the group C—O—C (12). Bands at 13.35 and 13.65μ have been stated to be characteristic of the 1,2-disubstituted benzene ring, which is present also in indoles (12). Bands were found at 12.15 and 12.4μ. Bands in the region of 11.8–12.6μ have been stated to indicate a 1,2,4-substituted benzene ring (17).

Saponification Equivalent.—To confirm the indication of the presence of an ester group, the hydrox-

[4] We wish to thank A. Turner of Smith, Kline and French Laboratories for carrying out the infrared determination, and S. Rump, of the same Laboratories, for aid in interpreting the infrared data.

amate test as outlined by Buckles and Thelen (18) was scaled down and applied to a 2.3-mg. sample of alkaloid. A positive test was obtained.

The saponification equivalent of the alkaloid was determined by a method described by Shriner and Fuson (19) scaled to accommodate a sample weighing 0.0207 Gm. The reagent used was alcoholic potassium hydroxide, and the reflux time was two hours. The saponification equivalent was 395.1, which agrees very closely with the calculated molecular weight (394.46), indicating the presence of one ester group in the molecule.

Test for Methylenedioxy Group.—Labat's test (20) was applied to 0.5 mg. of the alkaloid. A negative result was obtained. The same quantity of hydrastine hydrochloride gave a strongly positive test under the same conditions.

Color Test for Indole Alkaloids.—The sample was dissolved without heating in 1 cc. of concentrated hydrochloric acid. Four drops of 2% alcoholic *p*-dimethylaminobenzaldehyde were added, followed by 2 drops of 0.05% sodium nitrite solution. Results follow:

Control (no alkaloid)—colorless
Hydrastine hydrochloride—colorless
Yohimbine hydrochloride—pale greenish color
Vinca alkaloid—deep yellow color

Yohimbine has been reported to give a yellow to green color with this reagent. Other alkaloids containing the indole nucleus also give various colors (21).

Effect of Light.—The crystalline alkaloid, and also solutions of the alkaloid in alcohol or chloroform, gradually develop a yellow color when exposed to light. This phenomenon is being investigated further.

SUMMARY

1. The literature pertaining to the alkaloids of the genus *Vinca* (Fam. *Apocynaceae*) has been reviewed.

2. From *Vinca minor* L. a crystalline alkaloid has been isolated. Some physical and chemical properties of this alkaloid have been determined.

3. Since its properties are not identical with those of any other alkaloid isolated from the family *Apocynaceae*, the name *perivincine* is suggested for the crystalline alkaloid from *Vinca minor*.

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The Behavior of Organic Bases in Nitromethane*

By L. G. CHATTEN, M. PERNAROWSKI, and L. LEVI

When organic bases are dissolved in nitromethane they react with the solvent to form salts. This reaction occurs only if the pK_b (H_2O) value of the base is less than 11. Experimental evidence for such interaction is presented by isolation of a typical reaction product and by a study of the behavior of these compounds during nonaqueous titration.

IT WAS NOTED by Fritz and Fulda (1) who reported the titration of weak bases in acetic anhydride-nitromethane solvent systems, that a number of nitrogen heterocyclics could not be titrated successfully because of solvent interference which was attributed to the presence of acetic anhydride. Their solvent system offered optimum conditions for the quantitative determination of caffeine and attempts were made, in this laboratory, to extend the method to the differential analyses of codeine and caffeine in pharmaceutical preparations containing acetylsalicylic acid, phenacetin, caffeine, and codeine. However, the two alkaloids could not be recovered stoichiometrically. It is the purpose of this paper to explain these findings and to extend the investigation to a number of other organic bases.

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The authors wish to express their appreciation to Dr. W. H. Barnes of the National Research Council for determining the X-ray diffractions of the compounds and to Mr. Martin Butler for drawing the figures. Grateful acknowledgment is made for the helpful suggestions of Dr. L. I. Pugsley.

EXPERIMENTAL

Apparatus.—(*a*) Beckman Model G *p*H Meter, (*b*) Shielded Glass Electrode, Beckman No. 1190-80, (*c*) Sealed Calomel Electrode, Sleeve-type Beckman No. 1170-71, (*d*) Microburet, graduated to 0.01 ml., (*e*) Perkin-Elmer Model 21 Spectrophotometer.

Reagents and solutions.—(*a*) Nitromethane C. P. obtained from Fisher Scientific; (*b*) nitromethane, practical grade, obtained from British Drug Houses and Eastman Kodak; (*c*) acetic anhydride, A. C. S. grade; (*d*) glacial acetic acid, A. C. S. grade; (*e*) dioxane, A. C. S. grade; (*f*) 0.05 *N* perchloric acid in glacial acetic acid; (g) 0.05 *N* perchloric acid in dioxane.

Procedures.—(*a*) Milligram quantities of codeine and caffeine were dissolved in a 60-ml. nitromethane-acetic anhydride mixture (5 : 1). Titration was performed as outlined by Fritz and Fulda (1). The procedure was repeated omitting the acetic anhydride and using various volumes (30–60 ml.) of nitromethane as solvent.

(*b*) Two and seven-tenths milligrams of strychnine alkaloid were dissolved in 1 ml. chloroform, 10 ml. acetic anhydride and 50 ml. dioxane and titrated with redistilled nitromethane.

(*c*) *Preparation of aci-nitromethane addition products.* (*i*) 150 mg. of strychnine were dissolved in 1 L. of redistilled nitromethane (b. p. 100–101°) and allowed to stand at room temperature for thirty minutes. Distillation under vacuum was carried out at 1–2 mm. pressure and at a temperature of 22–27°. The volume was reduced to about 10 ml. and evaporated to dryness by passing a current of air over it. The residue was redissolved in 3 ml. of nitromethane and precipitated by the addition of carbon tetrachloride. The crystals were removed by filtration and the precipitation process was repeated three times. The isolated product was dried over phosphorous pentoxide. The creamy white, fluffy crystals showed a decomposition range of 250–260°. (*ii*) One hundred and forty milligrams of ephedrine were treated as described in (*i*) except that the residue was reprecipitated twice by dissolving in a minimum of nitromethane, adding an equal quantity of carbon tetrachloride and chilling in an ice bath. The isolated product was dried over phosphorous pentoxide to give white fluffy needles melting at 123.5 to 126°.

RESULTS AND DISCUSSION

The titration of caffeine in nitromethane-acetic anhydride as performed by Fritz and Fulda (1) was readily repeated in this laboratory. However, when mixtures of caffeine and codeine were titrated in this system, the end point observed for codeine was equal to about 60% of theoretical, whereas the end point observed for the total titration agreed, in all instances, with the theoretical (Fig. 1). When attempts were made to titrate the stronger base alone, in this system, a curve showing two inflection points was obtained. By replacing acetic anhydride with glacial acetic acid, similar results were obtained. Investigation also showed that maintaining the system at 0° did not prevent the phenomenon from occurring. A similar curve was observed when the alkaloid was titrated in nitromethane alone and Fig. 2 is a differential plot. In each instance, the curves (E vs. V) and the differential plot (dE/dV vs. V) are strikingly similar to those characterizing the differential titration of two compounds with appreciably different *p*K values. The first maximum in Fig. 2 corresponds to a recovery of approximately 64% codeine, whereas the second maximum represents an almost theoretical recovery. Using 50 ml. of nitromethane as solvent, the procedure was applied to a number of

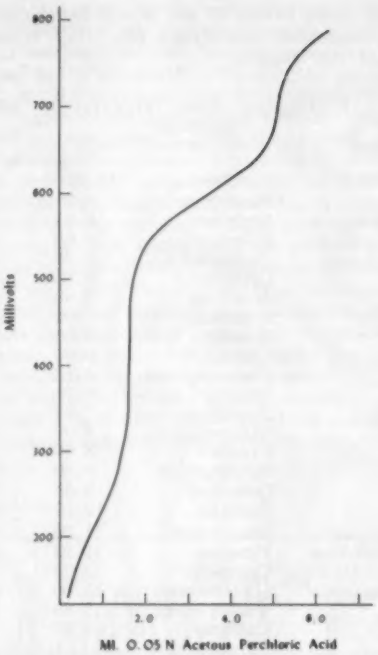


Fig. 1.—Differential titration of caffeine and codeine using a solvent system of acetic anhydride and nitromethane.

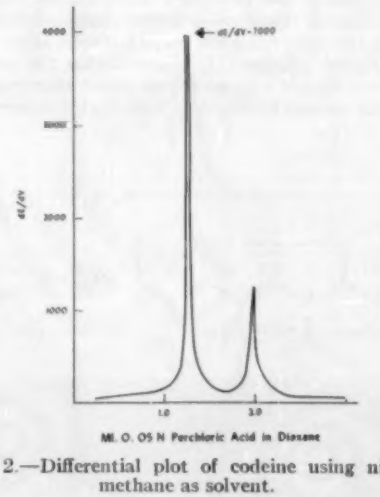


Fig. 2.—Differential plot of codeine using nitromethane as solvent.

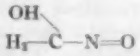
organic bases (Table I) and it was found that all those compounds exhibiting a pK_b (H_2O) below 11 showed two maxima.

TABLE I. ORGANIC BASES TITRATED IN NITROMETHANE[a]

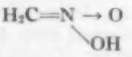
	Base	pK_b (H_2O)	Reference
Compounds exhibiting two dE/dV maxima	Benzedrine	4.29	(2)
	Atropine	4.35	(3)
	Ephedrine	4.68	(4)
	Epinephrine	5.80	(a)
	Cinchonine	5.85	(3)
	Aconitine	5.88	(3)
	Strychnine	6.00	(3)
	Brucine	6.04	(3)
	Codeine	6.05	(3)
	Physostigmine	6.12	(3)
	Solanine	6.66	(3)
	Narcotine	7.83	(3)
	Papaverine	8.07	(3)
	Pyridine	8.90	(3)
	Amidopyrine	9.30	(5)
	Quinoline	9.50	(3)
	Narceine	10.70	(3)
Compounds exhibiting one dE/dV maximum	Benzocaine	11.22	(3)
	Piperine	12.22	(3)
	Colchicine	12.35	(3)
	Nicotinamide	13.60	(6)
	Theobromine	13.89	(7)
	Caffeine	14.00	(7)

[a] Determined in this laboratory by the Method of Saunders and Srivastava (8).

Several authors (9–13) have demonstrated that the primary and secondary nitroparaffins exist as two isomers, the nitro- or normal form and the aci- form, the latter being less stable but more acidic than the former. Lucas (11) observed that the sodium salt was formed with aci-nitromethane when reacting it with sodium hydroxide. Corelli (12) determined the amount of aci- form in a particular sample by titrating it with alcoholic KOH, using Alkali Blau 6–B as indicator. He postulated the structure of the aci- form to be



whereas Taylor and Baker (10) visualized it as

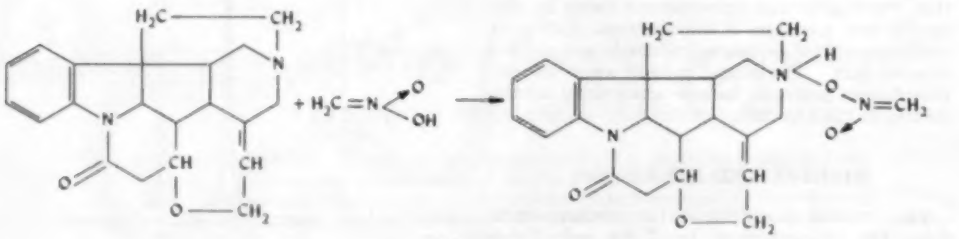


and the latter structure agrees with that reported by Wheland (13) and Lucas (11).

From the experimental work here reported, it would appear that the aci- form reacted with all bases whose pK_b (H_2O) was less than 11 to form a salt, which behaved, in nitromethane, similarly to those compounds whose pK_b value was greater than 11. Yet this process did not go to completion because of the weak ionization of the solvent (K nitromethane 2.6×10^{-11} and K aci 6×10^{-4}) (14) and the excess base being used in the procedure. Hence, in each instance two compounds were being titrated, (*viz.*) the original base and the salt, the neutralization of any unreacted base by perchloric acid being followed by titration of the salt. This process is illustrated for strychnine by Eq. I and II.

Further evidence for this mechanism was secured by increasing the amount of solvent, thereby providing sufficient aci-nitromethane to permit the process of salt formation to go to completion. It was found that 200 ml. of nitromethane provided sufficient aci- form to react completely with 25 mg. of codeine. Titration with perchloric acid produced a curve which showed only one maximum.

The occurrence of the reaction between the aci- form of the solvent and the stronger bases was further substantiated by the direct titration of mg.



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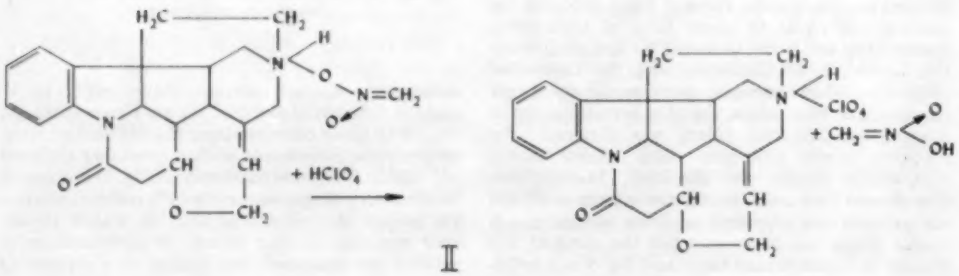

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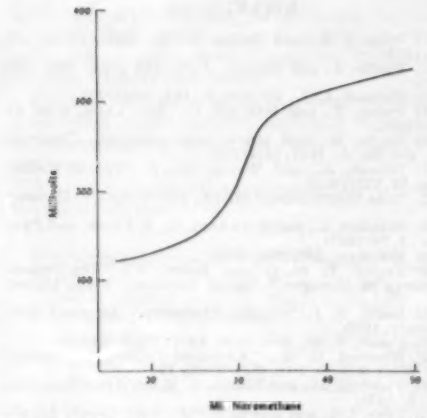

Fig. 3.—Titration curve of strychnine in a chloroform, acetic anhydride, dioxane solvent system.

quantities of strychnine with nitromethane (Fig. 3) and by isolation of the strychnine and the ephedrine aci-nitromethane addition product respectively as outlined in the procedure. The infrared spectrum and X-ray diffraction pattern of strychnine and its addition product are shown in Fig. 4 and 5. Similar differences were noted between the infrared spectrum of ephedrine and that of its addition product.

The titration curve of bases with pK_b (H_2O) values greater than 11 showed only one maximum. Attempts to titrate the weaker base colchicine with nitromethane were unsuccessful as no end point could be detected. These compounds apparently do not react with the solvent, thus, attempts to isolate a reaction product from a caffeine-nitromethane mixture, in accordance with the described procedure, were unsuccessful and unchanged alkaloid, only, was recovered from the system.

It is interesting to note in this connection that Fritz and Lisicki (15) who titrated nitromethane with sodium methoxide using butylamine as solvent,

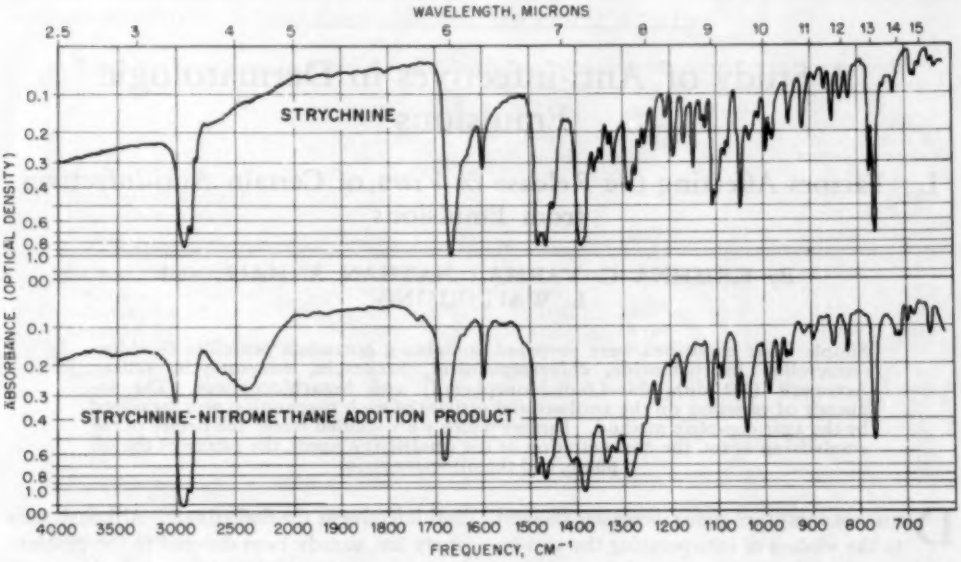

Fig. 4.—Infrared absorption spectra of strychnine and strychnine–nitromethane addition product.

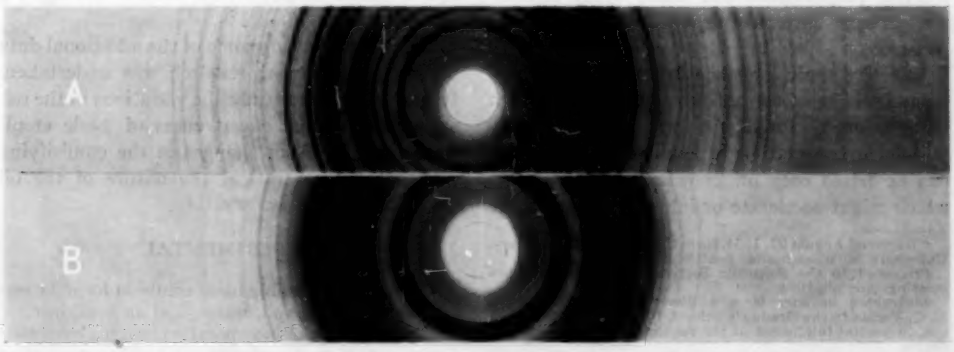

Fig. 5.—X-Ray diffraction pattern of (A) strychnine and (B) strychnine–nitromethane addition product.

obtained recoveries that were erratic and usually less than quantitative. This, they reasoned, was "due to partial decomposition of nitromethane caused by reaction with the butylamine solvent."

Ballczo (16) has described the disadvantages a solvent system may exhibit during titration and one of these is interaction with the substance to be determined. Evidently nitromethane possesses this property. Yet, the procedure outlined can still be used as a means of determining total alkaloidal content of mixtures of pure bases, provided each of these can be titrated in nitromethane with perchloric acid. As an example, triplicate determinations on mixtures of codeine and caffeine showed recoveries of total alkaloidal content equal to 99.86, 99.81, and 99.41%. These results led the authors to believe that by combining this procedure with a method (17) previously published, it would be possible to determine the caffeine as well as the codeine content of APC and C tablets. Investigation has shown, however, that the presence of phenacetin interferes with the assay of caffeine by nonaqueous titrimetry.

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A Study of Anti-infectives in Dermatologic Emulsions*

I. Factors Affecting the Release *in Vitro* of Certain Anti-infectives from Emulsions

By KRISHNA C. VARMA,† NATHAN A. HALL, and L. WAIT RISING

Simple basic emulsions were prepared containing potassium penicillin G, chlortetracycline hydrochloride, chloramphenicol, bacitracin, streptomycin sulfate, Actamer® (2,2′-dithio-bis-4,6-dichlorophenol) and hexachlorophene. The influence of variation on the antibacterial activity of each preparation was measured by the agar-cup-plate method. Factors which were studied were: the nature of the emulsifying agent, the concentration of the emulsifying agent, the nature of the oil phase, and the oil-water ratio.

DERMATOLOGISTS are giving increasing thought to the wisdom of incorporating the anti-infective agents they want to use into emulsion-type vehicles. These make pharmaceutically attractive preparations and under many circumstances appear to offer superior therapeutic performance.

Effective topical medication requires uninterrupted release of the active material to the area being treated. Intelligent formulation of a dermatologic preparation to achieve this objective can be based only on knowledge of the factors which might accelerate or inhibit transfer of the anti-infective at the desired rate. Although some study has already been devoted to the problem, the number of available chemicals both as antibacterials and carriers increases at such a pace that detailed investigations on their use lags far behind.

In order to develop some of the additional data needed the following research was undertaken. It was designed to study the variations in the relative antibacterial effectiveness of basic emulsions resulting from changes in the emulsifying agent, its concentration, the nature of the oil phase, and the oil-water ratio.

EXPERIMENTAL

The simplest possible basic emulsion formula was selected containing oil, water, and an emulsifier.

Antibacterials for incorporation into the emulsions were potassium penicillin G, chlortetracycline

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hydrochloride, chloramphenicol, bacitracin, streptomycin sulfate, Actamer® [2,2′-dithio-bis(4,6-dichlorophenol)] and hexachlorophene [2,2′-methylene-bis-(3,4,6-trichlorophenol)].

For each antibacterial agent a concentration was used which would produce sufficient inhibition to be measurable but which was considerably less than maximum.

A typical medicated emulsion prepared from the basic formula was:

Peanut oil	50	
Water	50	
Arlacel® 60	1.5	
Chlortetracycline hydrochloride	10	μg/ml.

Variations were made in the nature of the oils, emulsifying agents, and antibacterials. In addition varying concentrations of emulsifiers and antibacterials, and oil-water ratios were used. All emulsions were passed through a hand homogenizer.

The F. D. A. agar-cup-plate technique (1) was adapted to each formula and used to measure the antibacterial activity to the nearest 0.5 mm. *Staphylococcus aureus* was used as the test organism for penicillin, Actamer®, and hexachlorophene; *Sarcina lutea* for chlortetracycline and chloramphenicol; *Staphylococcus albus* for bacitracin; and *Bacillus subtilis* for streptomycin.

Influence of Emulsifying Agents.—A group of emulsifying agents was selected at random from the different classes for the preparation of emulsions of peanut oil containing the anti-infectives. The activity of each emulsion was compared to a simple aqueous solution for the water-soluble agents and to an emulsion with Tween® 80 for the water-insoluble agents (Table I).

To examine the effect of the concentration of the emulsifying agent upon the activity of the emulsions, five concentrations of Tween® 80 (6–10%), Span® 40 (1–1.5%), glyceryl monostearate–self-emulsifying (1–1.5%), and Arlacel® 60 (1–1.5%) were combined with the anti-infectives. These emulsifiers did not possess any activity of their own and their emulsions showed suitable release of the medicating agents. In no case did the variations in concentration of the emulsifying agent significantly affect the activity of the emulsion.

TABLE I.—INFLUENCE OF EMULSIFYING AGENTS ON ANTIBACTERIAL ACTIVITY OF EMULSIONS[a]

	Penicillin (10 units/ml.)	Chlortetracycline (10 μg/ml.)	Chloramphenicol (10 μg/ml.)	Bacitracin (10 units/ml.)	Streptomycin (15 μg/ml.)	Hexachlorophene (4.5 mg/ml.)	Actamer (4.5 mg/ml.)
Anionic							
Ammonium stearate	72	50	64	[b]	85	70	163
Magnesium stearate	61	0	64	0	0	63	74
Acacia	104	86	96	65	89	0	0
Triethanolamine	56	0	107	65[c]	133	144	170
Sodium sulforicinoleate[c]	104	50	114	155	130	119	130
Sodium lauryl sulfate[c]	108	91	146	[b]	133	126	122
Santomerse® #3[c]	85	102	164	159	148	141	141
Aerosol® OT[c]	96	48	122	116	119	100	100
Cationic							
Secal[c]	91	100	111	84	81	[b]	[b]
Zephiran®[c]	96	132	196	175	207	145	163
Nonionic							
Arlacel® 60	96	93	82	100	89	63	63
Span® 40	98	198	93	116	103	70	70
Tween® 80	100	102	89	110	103	100	100
Glycervl monostearate S. E.[d]	91	84	78	100	81	63	67
Miscellaneous							
Bentonite	100	45	86	0	0	0	74

[a] Comparative values based on aqueous solution as 100 for antibiotics and Tween® 80 emulsion as 100 for chlorinate phenols.
[b] Indistinct, not measurable.
[c] Unmedicated emulsions showed considerable activity.
[d] Some ionic activity may be shown by this agent depending upon the emulsifier used to make it self-emulsifying.

TABLE II.—EFFECT OF DIFFERENT OILS ON ANTIBACTERIAL ACTIVITY OF EMULSIONS

	Zone of Inhibition in mm.						
	Penicillin (5 units/ml.)	Chlortetracycline (20 μg/ml.)	Chloramphenicol (20 μg/ml.)	Bacitracin (10 units/ml.)	Streptomycin (15 μg/ml.)	Hexachlorophene (5 mg./ml.)	Actamer (5 mg/ml.)
Soya bean	22.5	22.0	20.5	14.5	13.0	13.0	13.5
Olive	23.0	22.5	20.5	15.0	13.0	13.5	11.5
Sesame	23.0	23.0	20.5	16.0	13.5	13.5	12.0
Castor	25.0	23.5	17.0	17.0	15.0	15.5	14.5
Cotton seed	22.0	22.5	20.5	15.5	13.0	13.0	12.5
Corn	22.0	22.0	20.5	14.5	13.0	13.0	12.5
Peanut	22.5	22.5	19.5	15.0	13.0	13.5	13.0
Lt. liquid pet.	23.0	22.5	21.0	10.5	13.0	14.0	12.5
Hvy. liquid pet.	21.5	22.0	20.0	14.0	12.0	14.0	12.5
Water solution	22.5	23.5	21.0	15.5	13.0	...	...

Influence of the Oil Phase.—A series of nine different oils was used in the preparation of the emulsions for testing. Because of the superiority of emulsions prepared with them, Arlacel® 60 was used as the emulsifying agent for the antibiotics and Tween® 80 for the chlorinated phenols (Table II).

To determine whether the concentration of oil would influence the activity of the emulsions, a series of peanut oil emulsions were prepared in which the oil-water ratio varied from 30:70 to 70:30. A slight decrease in activity was observed as the concentration of the oil was increased. This decrease in activity seemed to parallel the increase in viscosity of the emulsions.

SUMMARY

The choice of an emulsifying agent is important in the formulation of dermatologic emulsions but the concentration of the nonionic emulsifier over a limited range seems to have little effect upon the release of anti-infective agents. The selection of an oil only slightly affects the activity of emulsions prepared with it. Increasing concentrations of the peanut oil slightly decrease the activity of anti-infective emulsions under the experimental conditions.

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The Influence of the Rate of an Intermittent Administration upon the Lethal Dose of Digitalis Tincture in Pigeons*

By DANIEL P. N. TSAO

A study of the rate of administration of digitalis tincture in pigeons by a five-minute-interval injection over a wide range indicated that there is a relation between the lethal dose and rate of administration at the levels of from 0.0126 to 0.1661 ml./Kg./5 min. during the experimental time of from thirty to three hundred and sixty minutes. The lethal dose increases with the increase of the rate of administration but within the experimental time employed this linear increase was not considered to be significant.

THE INFLUENCE of the rate of administration on the lethal dose of digitalis tincture and pure glycosides has been studied with controversial reports (1–5). Bliss and Allmark (1) reported that with a twofold increase in the amount of tincture, the lethal dose increased by 15 per cent. Farah (2) indicated that qualitative and quantitative changes in the heart work of heart-lung preparations did not significantly influence either the lethal dose or average rate of uptake of G-strophanthin in heart-lung preparations of the dog. Maresh and Farah (3), using cats, showed that with high rates of continuous injection of digitoxin and other glycosides the lethal dose was high, but with decreasing rates the lethal dose fell until a kind of plateau could be reached at a very wide range of slow injections. Jacobsen and Larsen (4) mentioned that the lethal dose of digitalis tincture decreased with increasing infusion time in guinea pigs. Tsao, Farah, and Youngken (5) indicated that there was no significant change in the lethal dose of digitalis tincture at the levels of from 0.0024 to 0.0226 ml./Kg./min. during the experimental period of from forty-two to three hundred and forty minutes by a continuous injection method. Since the five-minute-interval injection is officially recognized in the U. S. P. XIV for the assay of digitalis, it was deemed important to investigate by the use of this method the effect of the rate of administration upon the lethal dose. The object of this study was to determine further relationships between the lethal dose of digitalis tincture in pigeons with the rate of administration and experimental time by using a five-minute-interval injection.

MATERIALS AND METHODS

Digitalis tincture was prepared from a U. S. P. Digitalis Reference Standard Powder according to the method mentioned by Tsao, *et al.* (5). The U. S. P. XIV bioassay procedure for digitalis (6) was employed with pentobarbital sodium[1] in place

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[1] Although more convenient than ether anesthesia, it has been noticed by Kaplan and Visscher (7) that under this condition the lethal dose is increased by 41–50%.

of anesthetic ether to provide a more convenient anesthesia. A 5-ml. microburet, calibrated to 0.01 ml., was used for the delivery of injections. The outlet of the buret was fitted with a No. 22 gage blunt needle by means of intravenous rubber tubing. The air bubbles were removed from the injection apparatus and a volume of specified diluted solution of digitalis tincture equivalent to 1 ml. per Kg. of the body weight of each pigeon was administered at five-minute intervals until the pigeons died of cardiac arrest. The following amounts of digitalis tincture were employed: 0.19, 0.25, 0.37, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, and 2.50 ml. In each case the tincture was diluted to 15 ml. with isotonic sodium chloride solution at the time of experiment.

Pigeons weighing 400–650 Gm. were given water *ad lib.* but were fasted from eighteen to twenty-four hours prior to use. Six pigeons were employed in each experiment as shown in Table I. The birds were anesthetized with an intramuscular injection of pentobarbital sodium (40 mg./Kg.). The alar vein was exposed and cannulated with a No. 22 gage blunt needle. The injection was administered at five-minute intervals.

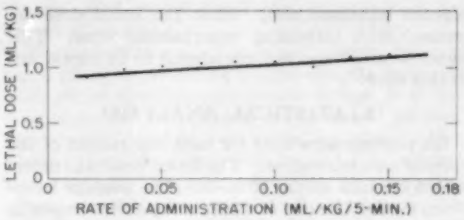

Figure 1.

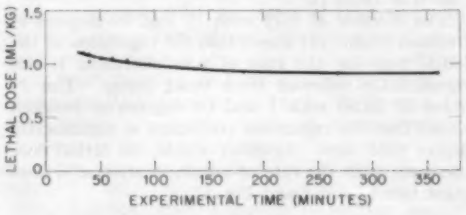

Figure 2.

TABLE I.—THE FIVE-MINUTE-INTERVAL INJECTION RATE OF THE ADMINISTRATION OF DIGITALIS TINCTURE AND THE LETHAL DOSE IN PIGEONS

Number of Animals (Pigeons)	Dilution (Tincture[a] to Isotonic NaCl Soln.)	Rate of Administration (ml./Kg./5 Min.)	Average Lethal Dose (ml./Kg.)	Experimental Time (Min.)
6	0.19:15	0.0126	0.9115 ± 0.0498	360.0 ± 18.6
6	0.25:15	0.0168	0.9106 ± 0.0277	268.0 ± 8.2
6	0.37:15	0.0248	0.9190 ± 0.0265	182.0 ± 5.4
6	0.50:15	0.0336	0.9612 ± 0.0351	143.0 ± 5.3
6	0.75:15	0.0502	0.9960 ± 0.0306	96.0 ± 3.4
6	1.00:15	0.0674	1.0341 ± 0.0225	74.5 ± 2.3
6	1.25:15	0.0832	1.0534 ± 0.0491	60.8 ± 3.3
6	1.50:15	0.1001	1.0507 ± 0.0391	50.5 ± 1.8
6	1.75:15	0.1172	1.0174 ± 0.0368	40.3 ± 1.6
6	2.00:15	0.1331	1.0849 ± 0.0396	39.0 ± 1.6
6	2.25:15	0.1499	1.0987 ± 0.0580	34.3 ± 2.1
6	2.50:15	0.1661	1.1047 ± 0.0490	30.2 ± 1.6

[a] Tincture of U. S. P. Digitalis Reference Standard.

TABLE II.—ANALYSIS OF VARIANCE

Source of Variation	D. F.	Sum of Squares	Mean Squares	F
Rate of administration	11	.337353	.030668	
Linear regression	1	.297006	.297007	28.98
Deviation from linearity	10	.040347	.004035	0.39
Error	60	.615007	.010250	
Total	71	.952360		

RESULTS

The results of the administration of the standard digitalis tincture by five-minute-interval injection into 72 pigeons are shown in Table I. In Fig. 1 and 2 the rates of administration and experimental time are plotted against the lethal dose, respectively. At the rate of 0.0674 ml./Kg./5 min. the lethal dose was 1.0341 ± 0.0225 ml./Kg. and is designed as a standard because it is the rate recognized in the U. S. P. XIV for the bioassay of digitalis. All the other lethal doses were compared with this one. At the rate of 0.1331 ml./Kg./5 min. with twofold increase in rate the lethal dose was 5% higher. At the rate of 0.0336 ml./Kg./5 min. the lethal dose was 7% lower while the rate of administration decreased twofold. Even at the slowest rate such as 0.0126 ml./Kg./5 min. with a sixfold decrease in rate the lethal dose was 12% lower, while during the fastest administration of 0.1661 ml./Kg./5 min. with a two and one-half fold increase in rate the lethal dose was 7% higher. This study shows that there is a relation between lethal dose of digitalis tincture and the rate of administration at the levels of from 0.0126 to 0.1661 ml./Kg./5 min. with experimental time of from thirty to three hundred and sixty minutes. As the rate of administration increased the lethal dose of digitalis tincture in

pigeons increased also; while the lethal dose decreased with increasing experimental time. This linear increase was not considered to be significant in this study.

STATISTICAL ANALYSIS[3]

Six pigeons were used for each experiment of the rates of administration. The lethal dose was determined for each of the 72 birds. The average lethal doses for the 12 rates of administration are shown in Table I. In statistical terms this study is a completely randomized experiment with 12 treatments and 6 replications. The analysis of variance is shown in Table II.

The *F*-value of 0.39 with 10 and 60 degrees of freedom (Table II) shows that the regression of the lethal dose on the rate of administration is not significantly different from being linear. The *F*-value of 28.98 with 1 and 60 degrees of freedom shows that the regression coefficient is significantly higher than zero. In other words, the lethal dose increases with the rate of administration at a constant rate (Table I and Fig. 1).

SUMMARY

A wide range of the five-minute intermittent rates of injection of digitalis upon its lethal dose in pigeons has been studied.

There is a relationship between the lethal dose of digitalis tincture in pigeons and the rate of administration at the levels of from 0.0126 to 0.1661 ml./Kg./5 min. The lethal dose increased with the increase in the rate of administration and decreased with increasing experimental time and logarithmically this increase could be significant at higher rates than employed in this study.

A statistical analysis of variance confirmed that the lethal dose increased with the rate of administration at a constant rate.

[3] The author is indebted to Dr. Jerome C. R. Li of the Mathematics Department of Oregon State College for statistical analysis.

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A Preliminary Study of the Hydrolytic Products of Gentiopicrin by Paper Chromatography*

By ARNOLD ALPERT† and EGIL RAMSTAD

Paper chromatography was used to study gentiopicrin and its hydrolytic products. In addition to glucose, four substances are formed during the hydrolysis of gentiopicrin with β-glucosidase.

GENTIOPICRIN, the glycoside of *Gentiana lutea* L., has resisted attempts at elucidation of its structure. In 1905, Tanret established the empirical formula as $C_{16}H_{20}O_9$ (1). The work of Asahina and his co-workers (2, 3) constitutes the bulk of positive information to date.[1] Along with a partial configuration for gentiopicrin Asahina suggested that its aglycone exists in three forms, which he termed protogentiogenin, *eu*gentiogenin, and mesogentiogenin. Protogentiogenin was the term applied to the aglycone as it exists in combination with the sugar component. Asahina contended that the substance which Tanret named gentiogenin, and which he thought constituted the aglycone part of the glycoside, was in reality a polymerization product, probably a dimer.

Asahina did not obtain any of the aglycones, except gentiogenin, in genuine form but as liquid impure mixtures from which crystalline hydrogenation products could be separated. Would it be possible directly to prove the existence of several forms of the aglycone from gentiopicrin? For such an investigation, paper partition chromatography seemed particularly advantageous.

EXPERIMENTAL

Preparation of Gentiopicrin.—A quantity of 3.8 Kg. of powdered, dried, stabilized gentian root[2] was percolated with alcohol in a repercolation setup; the alcoholic extract was evaporated *in vacuo* to 2 liters. After standing at 7° for four weeks,

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† Ensign Alpert is now on active duty with the U. S. Navy.

[1] After termination of this work, Kröte (11) published more data on gentiopicrin. On the basis of additional tests and infrared and ultraviolet spectra he proposes a definite formula for the glycoside.

[2] We are indebted to Professor H. Flück, of the Eidgenossische Technische Hochschule in Zurich for kindly supplying the stabilized dried gentian root.

the liquid was decanted from large quantities of sugars, then evaporated to 850 ml. The glycoside was partitioned countercurrently between water and a mixture of chloroform and ethanol (2 + 1) through 5 units. The chloroformic layers (3.5 liters) were evaporated *in vacuo* to dryness. The residue was dissolved in one liter of hot alcohol, the solution seeded with specially prepared gentiopicrin, and allowed to crystallize. Yield: 45 Gm. gentiopicrin.

After repeated recrystallization from absolute alcohol and drying *in vacuo* at 110°, the melting point (Kofler micromethod) was 176–177° (Tanret: anhydrous gentiopicrin 191°, hydrous gentiopicrin 120–121°). $[\alpha]_D^{25} = -200.0°$ (2% in water). [Messmer (4): anhydrous, $[\alpha]_D = -201.2°$; Klein (5): hydrous, $[\alpha]_D^{22} = -197.6°$.] The slightly yellow, orthorhombic crystals possessed the characteristic intense bitterness of gentiopicrin. Bridel's identity test (6) was positive. Hydrolysis gave a reducing sugar and a crystalline aglycone, gentiogenin, m. p. 182–184° (Kofler), with swelling (Asahina: 184°).

Small quantities of glucose clung tenaciously to the gentiopicrin and apparently explains the somewhat low melting point (12), but did in no way interfere with the following experiments. Tanret (1) has discussed difficulties encountered in obtaining exclusively the hydrous or the anhydrous form of gentiopicrin.

Chromatographic Detection and Identification of Gentiopicrin.—As a means of detecting and locating gentiopicrin on a chromatogram, the method of Bliss and Ramstad (7) was applied successfully. By use of emulsin as the hydrolyzing enzyme followed by aniline hydrogen phthalate as the detecting reagent, various materials containing gentiopicrin including crude alcoholic extracts of gentian root, crude gentiopicrin, and the purified glycoside itself were chromatographed. By testing the chromatograms with the aniline hydrogen phthalate solution, with and without the preceding enzymatic treatment, a single, brown spot was observed. Its R(glucose)-value ranged from 4.8 to 5.1 with an average of 4.9. Under ultraviolet light the spot was even more pronounced and detection could be so made where in ordinary light the spot was not readily visible.

Gentiogenin.—Only the gentiogenin form of the aglycone of gentiopicrin could be prepared. Attempts at preparation of eugentiogenin and mesogentiogenin according to the procedures outlined by Asahina were not successful. Chromatographic analysis of the fractions showed that they all consisted of a similar complex mixture.

Pure gentiogenin was prepared essentially according to Asahina's method (2). Under a microscope it appeared as small, slightly yellow, needle-shaped crystals, m. p. 182–184° (Kofler) (Asahina: 184°).

Chromatography of the Hydrolyzing Mixture of Gentiopicrin.—The descending chromatographic procedure used was that described by Jensen (10) involving a benzene-chloroform-formamide solvent system. Use of reagent sprays for detection of the spots was not necessary except for locating the gentiopicrin, since all spots were visible under ultraviolet light. In cases where further developing sprays had to be used, the chromatograms were dried overnight at room temperature in place of oven-drying. Allowance of one to four hours for saturation of the chamber tended to reduce the degree of streaking.

The hydrolyzing mixture of gentiopicrin was prepared (2, 3) by dissolving one gram of the glycoside in 20 ml. of distilled water and acidifying with one drop of acetic acid. Thirty milligrams of purified, highly active emulsin were added and the mixture allowed to stand at room temperature. At selected time intervals, approximately 15 μg. of the hydrolyzing mixture were applied to a chromatographic strip freshly impregnated with formamide, and the strip was developed and dried according to Jensen (10).

A series of twelve chromatograms was obtained representing hydrolysis durations of thirty minutes up to seventy-two hours. Including the spot appearing at the site of application, a total of four spots was observed and designated, in order of increasing R_f values, as spots A, B, C, and D. Spot B was not clearly visible on chromatograms representing the first four hours of hydrolysis. The R_f values were: A = 0, B = 0.02, C = 0.08, D = 0.61.

The fact that spot B was different from spot A was shown in two ways: Prolongation of development time, up to twenty-four hours, caused B to travel additional distances and it thereby became distinctly separated from A. Two-dimensional paper chromatography showed a lateral movement for B with none indicated for A.

Chromatography of the Available Hydrolytic Products of Gentiopicrin.—Gentiopicrin, glucose, and gentiogenin were chromatographed individually in the benzene-chloroform-formamide solvent system under conditions the same as those for the hydrolyzing mixture of the glycoside. The location of glucose was established with aniline hydrogen phthalate and the gentiopicrin by means of the method of Bliss and Ramstad (7). The gentiogenin spot was visible under ultraviolet light without recourse to the use of reagents. Even after prolonged development of the chromatogram, each of the three materials showed no displacement from the site of application, i. e., they all have the R_f value of zero.

Control experiments showed that neither a solution of gentiopicrin with acetic acid nor a solution of emulsin alone causes the appearance of the spots originating from the hydrolyzing mixture.

DISCUSSION

In his work Asahina proposed that the genuine aglycone, protogentiogenin, is stable only when combined with glucose and that hydrolysis first gives rise to mesogentiogenin, which, in turn, gives eugentiogenin and Tanret's gentiogenin, the dimer. There are, then, five compounds to be expected in a hydrolyzing solution of gentiopicrin, namely, the glycoside itself, glucose, gentiogenin, eugentiogenin, and mesogentiogenin. Paper partition chromatography demonstrates the presence of three unidentified substances in a hydrolyzing mixture of gentiopicrin. Since spots B, C, and D correspond to none of the available hydrolytic products and since, up to date, only two other substances have been derived from hydrolysis of gentiopicrin, it would seem that an additional unsuspected substance has been formed.

CONCLUSIONS

1. Paper partition chromatography in conjunction with emulsin provides a method for detection and identification of gentiopicrin (R_{gluc} value of gentiopicrin is 4.9 with solvent: butanol 4, acetic acid 1, water 5).

2. Paper partition chromatography of a hydrolyzing mixture of gentiopicrin has confirmed Asahina's postulate that gentiopicrin upon hydrolysis furnishes an aglycone existing in several forms.

3. Paper partition chromatography of a hydrolyzing mixture of gentiopicrin indicates the existence of a hitherto unreported hydrolytic product.

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The Effect of *p*H Variations of Ointment Bases on the Local Anesthetic Activity of Incorporated Ethyl Aminobenzoate*

I. Hydrophilic Ointment U. S. P.

By V. N. BHATIA and R. H. BARBER†

Ointments prepared by incorporating five per cent ethyl aminobenzoate in Hydrophilic Ointment U. S. P. were buffered at different *p*H values ranging from 3.5 to 10. The local anesthetic activity of these ointments was compared by the "rat-tail" method of measuring analgesia. The optimum activity was found to be close to the *p*H of rat skin and decreased markedly in ointments having a higher or lower *p*H.

DURING the last ten years a considerable amount of work has been done to study the various aspects of absorption of medicaments through the intact skin and their resultant therapeutic activity. However, so far no systematic attempt has been made to determine whether the *p*H of ointment bases has any effect on the penetration and subsequent action of the incorporated medicinal substance. The aim of this investigation was to study this aspect of the problem.

Lucas and Guth (1) have recently shown that a method similar to that described by D'Amour and Smith (2) in 1941, could be used to study the penetration of local anesthetics through the skin. It was, therefore, decided to study the local anesthetic activity of ethyl aminobenzoate ointments, prepared from various types of buffered ointment bases; as this would not only shed some light on the action of buffered local anesthetic ointments but also on the larger problem of the relationship between the *p*H of ointment bases and the absorption of incorporated medicinal substances. This paper deals with the first part of this study in which the ointment base used was hydrophilic ointment U. S. P.

The method used consists basically in applying heat to a small area on rat tails before and after the application of local anesthetic ointments and noting the difference in time that it takes for the animal to perceive pain, the perception of pain being indicated by the characteristic twitch of the tail.

EXPERIMENTAL

Five per cent ethyl aminobenzoate ointments in hydrophilic ointment U. S. P. were prepared and buffered at *p*H 3.5, 4, 5, 6, 6.5, 7, 8, 9, and 10. The buffering mixtures used were the Clark and Lubs buffers described in the U. S. P. XIV.

Fifty female albino rats, weighing 140–160 Gm., were used in this study. Only female rats were used because preliminary studies revealed that they responded more uniformly than the males. The animals were divided in groups of ten animals each. At least one group was used for each *p*H and the animals were allowed to rest for one week before being used for a second set of readings.

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The heat source consisted of a sealed beam headlight, mounted on a ringstand and directed downward. Located immediately below the headlight was a lens to focus the light to a point. The light was operated by a six-volt storage battery to which it was connected through a switch and a rheostat. In using this device the rat tail was placed on a marked spot (where the light was focused). The light was then switched on and at the same time a stop watch was started. As soon as the rat felt the pain caused by the heat and twitched its tail the elapsed time was noted. Preliminary experiments were conducted and light intensity was so adjusted that the untreated animals responded in approximately three seconds. This interval was found to be long enough for accurate timing without being so long as to make the readings inaccurate due to movements of the tail not caused by the experiment. In order that the response be uniform and constant, it was necessary first to train the animals by repeated exposures at intervals of a few days.

In taking the readings, a group of animals was placed in rat holders so that their tails protruded through slots. An area extending $1^1/_2$ in. from the tip of the tail was selected as the site for the application of ointments. This area was first painted black with a charcoal paste to ensure maximum absorption of heat. As soon as the charcoal dried it was placed under the light and the normal reaction time for each rat was determined as described above. Then the charcoal was removed with a damp cloth, the tail dried and uniformly covered with one gram of the ointment being tested. The ointment was allowed to remain in contact for one hour. At the end of this period the ointment was removed and the tail was again painted black with the charcoal paste. After the charcoal had dried the tail was placed under the light and the second reaction time was determined. Any increase in the reaction time would now depend on the amount of the local anesthetic absorbed through the skin.

The results are listed in Table I and graphically shown in Fig. 1.

The results were statistically evaluated by the calculation of student "t" values. The differences between the results at various hydrogen ion concentrations were found to be statistically significant with the exception of *p*H 6.5 and 7. The difference in the results at these two *p*H values showed no statistical significance.

DISCUSSION AND CONCLUSIONS

1. There is a considerable variation in the absorption and consequent local anesthetic action of ethyl aminobenzoate when it is incorporated in hydrophilic ointment buffered at varying *p*H values.

2. The maximum local anesthetic activity seems to occur at *p*H 6 and 7 and decreases sharply when the *p*H is varied.

3. The *p*H of the rat skin is approximately 6.48 (3). From the results of this study it appears that at least under these experimental conditions the maximum local anesthetic action will take place if the ointment is buffered at or near the *p*H of the skin.

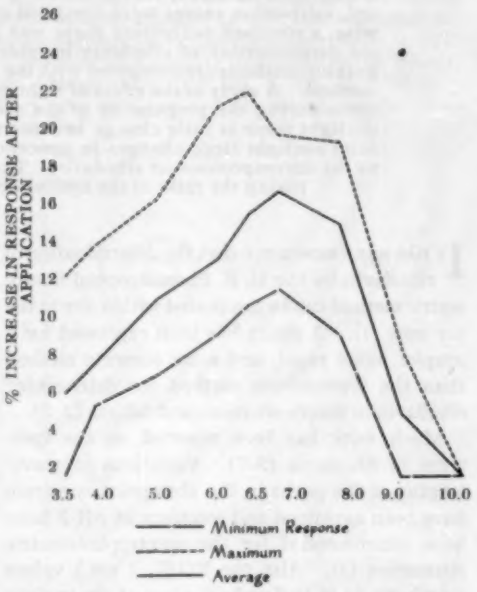

Fig. 1.— *p*H response curve

TABLE I.—THE MINIMUM, MAXIMUM, AND AVERAGE PER CENT INCREASE IN RESPONSE TIME AFTER APPLICATION OF 5% ETHYL AMINOBENZOATE IN HYDROPHILIC OINTMENT U. S. P.

*p*H of Ointment	Minimum, %	Maximum, %	Average, %
3.5	0.0	13.0	5.5
4.0	4.3	15.0	7.6
5.0	6.6	18.1	11.7
6.0	9.3	24.0	14.2
6.5	11.0	25.0	17.1
7.0	12.1	22.2	18.6
8.0	9.4	21.8	16.6
9.0	0.0	8.3	4.1
10.0	0.0	0.0	0.0

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Determination of Riboflavin by Light Absorption and Polarographic Methods*

By ARTHUR J. ZIMMER and C. LEE HUYCK

Using the Beckman spectrophotometer and the U. S. P. riboflavin reference standard, calibration curves were compiled in both visible and ultraviolet light. Likewise, a standard calibration curve was prepared on the polarograph. Results of the determination of riboflavin in tablets by the spectrophotometric and polarographic methods are compared with the results obtained by the official fluorometric method. A study of the effect of light on the absorbance of acid solutions of riboflavin during the preparation of the samples indicates that in prolonged diffused daylight there is little change in concentration of riboflavin. In prolonged artificial sunlight large changes in concentration are recorded in acid solutions due to the decomposition of riboflavin. The changes can readily be detected by comparing the ratio of the optical density at 265 and 270 millimicrons.

IT HAS BEEN REPORTED that the determination of riboflavin by the U. S. Pharmacopeial fluorometric method can be duplicated within five to ten per cent (1). A desire has been expressed for a simpler, more rapid, and more accurate method than the fluorometric method for determining riboflavin in simple solutions and tablets (2, 3).

Much work has been reported on the spectrum of riboflavin (2–7). Variations in wavelengths at the peaks in the absorption spectrum have been explained and solutions at *p*H 3 have been recommended for the spectrophotometric estimation (2). Also the E(1%, 1 cm.) values for riboflavin in buffered solutions at the various peaks were reported (2). There has been no report however of the use of the spectrophotometric method for the direct determination of riboflavin in tablets and other pharmaceutical preparations.

The polarographic method for the determination of riboflavin has been suggested (8) and it was recently reported for the determination of riboflavin in tablets (3). In this work solutions of riboflavin at *p*H 7.38 containing urea were polarographed. A precision of plus or minus 2 per cent was reported for the method. The polarographic method for the determination of riboflavin in partially purified riboflavin, solubilized riboflavin, and vitamin mixtures containing riboflavin by a standard addition method at *p*H 2.8 has also been reported (9).

The purpose of this investigation is to determine the feasibility of the spectrophotometric method for the direct determination of riboflavin in tablets and solutions and to compare the results with the results obtained by the polarographic and the U. S. P. fluorometric methods.

EXPERIMENTAL

Materials.—A sample of U. S. P. Reference Standard Riboflavin dried to constant weight at 105° was used for the preparation of the standard light absorption curves and the polarographic calibration curves. Riboflavin labeled U. S. P. was used in all the other investigations. Analytical grade glacial acetic acid was diluted with distilled water to make the 0.02 *N* acetic acid.

Apparatus.—A Beckman Model DU spectrophotometer and the Coleman Junior spectrophotometer were used for measuring light absorption in the preparation of the standard calibration curves in the ultraviolet and the visible regions of the spectrum. A Beckman Model G *p*H meter was used to determine the *p*H of the solutions. The Sargent Model III polarograph was used to make the polarographic measurements. The capillary has the following characteristics: $M^{2/3}t^{1/6}$ was 1.7 $mg.^{2/3}$ $sec.^{1/6}$ The height of the mercury column was 35 cm. The Pfalz and Bauer fluorometer was used to make the fluorometric measurements as directed in the U. S. Pharmacopeia XIV (10).

Preparation of the Calibration Curves.—Calibration curves for the spectrophotometric method were prepared with the Beckman spectrophotometer at wavelengths 267, 270, and 445 mμ. The calibration curve shown in Fig. 1 at 440 mμ was prepared with the Coleman Junior spectrophotometer. The wavelength 440 mμ was selected arbitrarily since no sharp peak is obtained with this instrument in this region as is the case with the Beckman instrument. Stock solutions were made according to the directions in the U. S. Pharmacopeia XIV (10), using Reference Standard Riboflavin. Aliquot portions were diluted to exactly 100 cc. at 25° with 0.02 *N* acetic acid. The *p*H of the solution was 3.4. The dilutions gave a range of concentrations from 0.5 to 10 μg./cc. Absorbance readings were taken on the spectrophotometers at the various wavelengths using 0.02 *N* acetic acid as a blank. The average of three readings of the instrument was taken for each point. Square quartz cells 1.00 ± 0.001 cm. were used in the Beckman spectrophotometer. Round

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50-cc. cells matched to within 1% transmission were used in the Coleman Junior spectrophotometer. To determine the effect of irradiation of ultraviolet light by the instrument, samples at various concentrations were allowed to stand for as long as fifteen minutes at the wavelengths used. No change in absorbance could be detected. Other samples in quartz cells were placed six inches away from the ultraviolet lamp for twenty minutes with no detectable change in absorption at the wavelengths used. The concentrations used were 2, 3, and 5 μg./cc. The calibration curves are shown in Fig. 1.

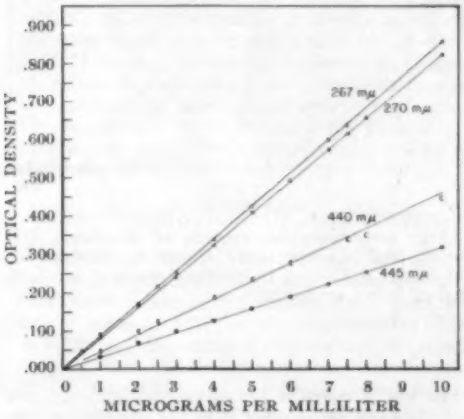

Fig. 1.—Calibration curves of riboflavin at various wavelengths. Points on curves 267, 270, and 445 mμ determined on Beckman spectrophotometer. Points on curve 440 mμ determined on the Coleman Junior spectrophotometer.

Although the curve at 440 mμ is drawn as a straight line, it actually is slightly curved and falls off in the higher concentrations. Deviation in the curve up to 7.5 μg./cc. may be largely due to instrumental error. However, in the range of concentrations from 10 to 25 μg./cc., the solutions of riboflavin failed to follow Beer's law owing to the fluorescence of the riboflavin. An additional calibration curve not shown in Fig. 1 was prepared on a Fisher electrophotometer using the 425 mμ filter. This curve was not a straight line in the range of concentrations covered in Fig. 1. The readings, however, were reproducible. Matched rectangular 10-cm. cells were used with this instrument.

In the polarographic method potassium chloride was used as the supporting electrolyte. Solutions were prepared by placing aliquot portions of the U. S. P. riboflavin stock solution in 100-cc. volumetric flasks with sufficient potassium chloride to make a tenth normal solution. Then 0.02 *N* acetic acid was added to make 100 cc. at 25°. Concentrations for the polarograph ranged from 10 to 100 μg./cc. The solutions were placed in an *H* type cell originally designed by Lingane and Laitenen (11) but modified by replacing the side gas arm with a capillary tube from the bottom of the electrolysis vessel. Oxygen was removed by bubbling purified nitrogen through the solution by means of the capillary attached to the bottom of the vessel. Well-defined curves were obtained when the solutions were polarographed from 0 to 1.5 v. using the saturated calomel electrode as a reference. The temperature was maintained at 25° ± 0.1°. The residual current was determined by polarographing a solution of the supporting electrolyte in 0.02 *N* acetic acid. The height of the wave was measured at 0.4 v. by subtracting the height of the residual current wave at 0.4 v. from the total height of the curve. The reading so obtained in mm. was converted to μa. by multiplying by the conversion factor 0.0122 μa. per mm. A sensitivity setting of 2 was used in all the determinations. In Fig. 2, the value of the diffusion current in μa. is plotted against the concentration.

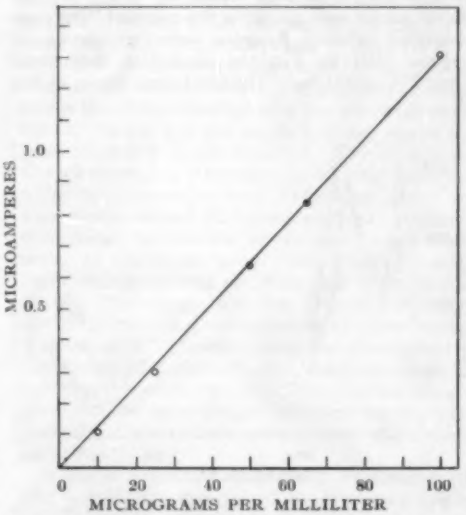

Fig. 2.—Calibration curve of riboflavin on the polarograph.

Effect of Light on the Routine Analysis of Riboflavin.—The decomposition of riboflavin by light is affected by various conditions such as *p*H, presence of oxygen, and temperature. Solutions at low *p*H are generally more stable than neutral or basic solutions. All solutions were prepared with 0.02 *N* acetic acid at *p*H 3.4 at 25°. Solutions of riboflavin were determined on the spectrophotometer after twenty-four hours exposure to the daylight of the laboratory. Changes that were noted in the absorption readings were within the limits of error of the instrument.

After the solutions were allowed to stand three or four days the readings were invariably lowered by a small but significant amount. To investigate this, two series of three solutions containing 2, 3, and 5 μg./cc. of riboflavin respectively were prepared. Series I was kept in the dark at room temperature and exposed to light only long enough to remove portions for analysis. Series II was kept out in the laboratory for twenty-four hours. No change in absorption was detected in either case at 265, 267, 270, and 440 mμ wavelengths. For the next twenty-four hours, Series II was kept in a constant light room at 100 candle power light intensity from the

General Electric daylight fluorescent lamps at 22°. This light is commonly termed "artificial daylight." The series showed a decrease in the absorption amounting to 20% at 270 mμ and 60% at 440 mμ wavelengths. After forty-eight hours exposure, absorption at 270 mμ decreased 60% and at 440 mμ the decrease was slightly more than 60%. However, the absorption at 267 mμ had decreased only 32%. The solutions of Series I kept in the dark showed no change in the absorption readings after three days. After fifteen days, these solutions showed a decrease in the absorption readings of less than 5% in all cases.

The shift in the peaks of the absorption spectrum of riboflavin in 0.02 *N* acetic acid at *p*H 3.4 under controlled light and temperature conditions was determined. Solutions containing 5 μg. of riboflavin per cc. were placed in the constant light room described above. Samples were withdrawn at regular intervals and the absorption determined from 214 to 500 mμ. The results are shown in Fig. 3.

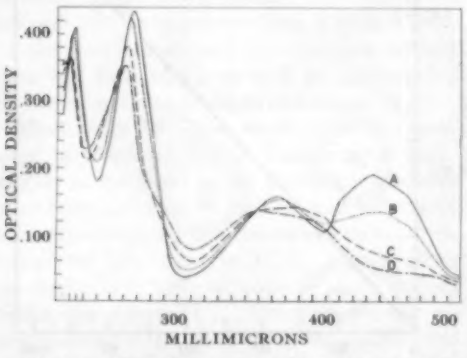

Fig. 3.—Absorption spectra of riboflavin 5μg./cc. 0.02 *N* acetic acid. Curve A, 0 hours, B, 2.5 hours, C, 8 hours; D. 20 hours.

Upon exposure to visible light the peak at 267 mμ was found to shift to 260 mμ. This shift begins in solutions containing 5 μg./cc. in less than three-hours exposure to artificial daylight. The first noticeable change is a lowering of the absorption on the higher wave-length side of the peak. Therefore, the reading of the absorption at 270 mμ decreases more rapidly than the reading at 267 mμ. For freshly prepared solutions the ratio of absorption at 265 mμ to 267 mμ is 1.014 ± 0.007. On exposure to light and on standing this ratio increases. After exposure to two and a half hours of artificial daylight the ratio shifted from 1.01 to 1.17 with an apparent decrease of 5% in the concentration calculated from the absorption at 267 mμ. If the concentration is calculated from the absorption at 270 mμ, the apparent decrease is almost 15%.

Solutions containing 100 μg. riboflavin/cc. 0.02 *N* acetic acid were irradiated at measured periods of time in the same manner as the dilute solutions. These solutions were diluted 1:20 with 0.02 *N* acetic acid and absorption was determined from 215–400 mμ. Readings were taken every 5 mμ except at the maximum absorption or at peaks where readings were taken at 1 or 2-mμ intervals. The results are shown in Fig. 4.

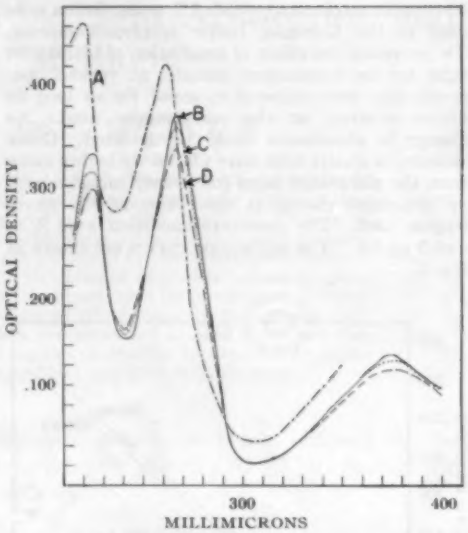

Fig. 4.—Absorption spectra of riboflavin 100 μg/cc. 0.02 *N* acetic acid. Curve A, Daylight for 30 days; B, C, and D, artificial sunlight for 3, 8, and 12 hours, respectively.

In Fig. 4 curve *A* represents the nonirradiated solution and is included here for comparison. It is noted that the shift in the wavelength of the peaks at 224 and 267 mμ of curve *A* corresponds to the shift after twelve hours of irradiation of the more dilute solutions of riboflavin shown in Fig. 3. However, the absorption shown in curve *A* of Fig. 4 is considerably higher at 220 and 260 mμ than that of the solution shown in Fig. 3 after twelve hours. From the absorption at 267 and 440 mμ, the concentration of riboflavin is 2.7 μg./cc. and 3.9 μg./cc. respectively compared to 1.8 μg./cc. when determined by the fluorometric method.

Curves *B*, *C*, and *D* of Fig. 4 show that the change in the ultraviolet absorption spectra upon irradiation with visible light is not as pronounced in concentrated solutions as it is in the more dilute solutions. After twelve hours of exposure to light of the same intensity as used for the solutions represented in Fig. 3, the shift at 224 mμ is not noticeable but the peak at 267 mμ has shifted to 266 mμ. The per cent decrease in absorption at 267 mμ after three hours is 10.6; after eight hours it is 13.3 and after twelve hours it is 18.8. After twelve hours of irradiation of the more dilute solution, the absorption at 267 mμ decreased 30%. The peak at 375 mμ did not shift, but showed a decrease in absorption comparable to the decrease in the absorption of the dilute solution at 375 mμ.

Acetic acid solution 0.02 *N* at *p*H 3.4 containing 52 and 100 mg. of riboflavin per liter stored at 4° in the dark for a period of one and two months respectively and a portion of the latter solution containing 100 mg. per liter exposed to daylight for four months, were determined spectrophotometrically and fluorometrically. The results are summarized in Table I.

TABLE I.—RIBOFLAVIN MG./L. IN 0.02 *N* ACETIC ACID AT *p*H 3.4 STORED IN LIGHT AND DARK

Sample Stored	Orig. Concn.	Fluorometric	Spectrophotometric at 270 mμ	267 mμ	440 mμ
Dark for 2 mo. 4°C.	100	91	92	96	98
Dark for 1 mo. 4°C.	52	53	50	50	51
Light for 4 mo. 25°C.	100	37	61	78	58

The agreement of the results for solutions stored at 4° in the dark is within the limits of error of the methods used. The results at 270 mμ agree with the fluorometric method better than the results at 267 and 440 mμ. However, the results on the sample exposed to light show a wider variation than can be explained by experimental error. The ratio of the absorption at 265 to 270 mμ of the first two solutions is 1.032 and 1.038 respectively and 1.30 for the third sample.

DETERMINATION OF RIBOFLAVIN

Solutions of Riboflavin U. S. P. were prepared by dissolving a weighed amount in a definite volume of 0.02 *N* acetic acid. This solution was diluted with 0.02 *N* acetic acid to give the concentration shown in Table II. Absorption at 265, 267 and 270 mμ was determined on the Beckman spectrophotometer and at 440 mμ on the Coleman Junior spectrophotometer. The absorption at 267 and 270 mμ according to the calibration curves given in Fig. 1 gave identical concentrations of riboflavin. Since the absorption readings at 270 mμ are more sensitive to changes in concentration than those at 267 mμ owing to decomposition of riboflavin by light, the calibration curve at 270 mμ was used for all determinations in this region. The curve at 440 mμ with the Coleman Junior spectrophotometer was used for the determination in the visible region. The results of the determination of riboflavin are shown in Table II.

TABLE II.—SPECTROPHOTOMETRIC DETERMINATION OF RIBOFLAVIN μG./CC. IN 0.02 *N* ACETIC ACID AT *p*H 3.4

Theoret.	No. Determ.	Av. Range	270 mμ	Av. Range	440 mμ
10	4	0.8	9.95	0.3	9.42
8	1	...	8.00	...	...
6	1	...	6.00	...	...
5	6	0.25	5.00	0.2	5.06
3	2	0.00	3.00	0.05	3.12
2	4	0.05	2.03	0.20	2.10

The theoretical concentrations found in the first column of Table II are calculated from the weighed amounts of riboflavin used in making the solutions. In the second column, the number of determinations made on each solution is given. The range shown in the third and fifth columns is the difference between the highest value and the lowest value in the series of determinations. The average of the series is shown in the designated column. The agreement of the results at 270 mμ with the theoretical value is good. Using the Coleman Junior spectrophotometer at 440 mμ the agreement with the theoretical is not as good as the agreement with the Beckman spectrophotometer at 270 mμ, although the largest deviation occurs at the concentration where the calibration curve falls off. In the range of absorption corresponding to the concentration covered by the calibration curve at 440 mμ in Fig. 1, the instrumental error is high but the agreement of the results shown in Table II at this wave length was felt to be satisfactory.

DETERMINATION OF RIBOFLAVIN IN TABLETS

In the usual procedure, twenty tablets were weighed and crushed. An amount of powder equivalent to one tablet was weighed to the fourth place and placed in a small beaker. Thirty to 40 cc. of 0.02 *N* acetic acid was added and the mixture was warmed on a water bath. If starch was present, it was found expedient to keep the temperature below 50° to prevent the swelling of the starch and subsequent difficulty in filtration. The solution was filtered through a Whatman No. 40 filter paper into a 100-cc. volumetric flask. The filter paper was washed with warm 0.02 *N* acetic acid until no fluorescence could be detected on the paper when placed under an ultraviolet lamp. No difficulty was encountered in washing the filter paper free of riboflavin. The solution was then diluted with 0.02 *N* acetic acid to give a concentration of approximately 5 μg/cc. at 25° and absorption was determined on the spectrophotometers. The total elapsed time was approximately one hour from the weighing of the tablets to the reading of the spectrophotometers. Several determinations were carried on simultaneously.

A blank containing magnesium stearate, starch, and lactose was determined by the spectrophotometric method by extracting a portion of the base with 0.02 *N* acetic acid. It was found that these materials did not contribute to the absorption of the filtered solution at 270 mμ or 440 mμ. The results of the determinations of several samples of tablets is shown in Table III.

With the exception of the first sample, the tablets were taken from stock and reassayed by the U. S. P. Fluorometric Method. Sample No. 1 is a small batch of a tablet trituration made in the laboratory.

From Table III, it may be seen that the precision of the spectrophotometric method is high. The agreement between the results of the fluorometric method and the visible light absorption method is better than the agreement between the fluorometric and the ultraviolet light absorption method. The fluorometric method generally gave higher values than either the spectrophotometric or the polarographic methods. In the polarographic method, the tablet debris was not removed from the solution by filtration since it did not interfere with the determination. However, there may be cases when filtration is necessary depending upon the materials used in the tablet being examined.

All the determinations are within the U. S. P. limits of labeled amounts of riboflavin per tablet. The low values obtained at 270 mμ are difficult to explain, since interference from inert materials in the tablet would tend to give higher absorption and

TABLE III.—RIBOFLAVIN MG./TABLET BY FLUOROMETRIC, SPECTROPHOTOMETRIC AND POLAROGRAPHIC METHODS

No.	Fluorometric	No. Determ.	Av. Range	270 mμ	Av. Range	440 mμ	Polarographic
1	4[a]	3	0.2	3.82	0.15	3.98	...
2	5.27	3	0.10	4.93	0.05	4.98	...
3	5.26	5	0.00	4.90	0.03	5.30	5.06[b]
4	5.35	5	0.05	5.08	0.05	5.35	...
5	5.40	2	0.00	5.10	0.00	5.25	5.00[c]
6	5.50	2	0.00	5.10	0.00	5.25	5.75[c]

[a] Calculated from added amounts.
[b] Average of six determinations.
[c] Average of two determinations.

higher than normal results. Absorption of riboflavin by filter paper was ruled out since the examination of the paper under the ultraviolet lamp after washing showed no fluorescence and the same dilution was used for the reading at 440 mμ. Furthermore, in sample No. 3 the same filter paper was used for three determinations while separate filter papers were used in the other two determinations with no apparent deviation. Since the total time necessary for the complete determination was only one hour, and since the solutions were protected from light; the decomposition of the riboflavin by light is not a probable explanation.

A liquid polyvitamin emulsion containing riboflavin was examined. It was found that the dispersing agent interfered with the spectrophotometric method, but the fluorometric and polarographic methods were applicable. The emulsion was found to contain 1.10 mg. riboflavin per 5 cc. by the fluorometric method and 1.12 mg. per 5 cc. by the polarographic method. For the polarographic method, the emulsion was diluted one to ten with 0.02 *N* acetic acid containing 0.1 *N* potassium chloride and polarographed without further separation. Identical results were obtained in duplicate determinations.

The riboflavin in the multivitamin preparation containing minerals could not be determined directly by either the spectrophotometric or the polarographic methods because of interference by one or more of the ingredients.

SUMMARY

1. Standard curves were prepared from Reference Standard Riboflavin for spectrophotometric and polarographic methods of determination.

2. The effect of light on the determination of riboflavin in pharmaceutical preparations in which the concentration of riboflavin is at least 2 μg./cc. 0.02 *N* acetic acid solution at *p*H 3.4 is negligible, provided ordinary care is taken. The decomposition of riboflavin by light may be detected by comparing the ratios of the absorption of the exposed solutions at 265 to 270 mμ with the ratios of the absorption of the freshly prepared solutions at the same wavelengths.

3. The spectrophotometric method for determining riboflavin in the ultraviolet and visible regions was found to be rapid and precise in the absence of interfering substances. The standard curve at 270 mμ wavelength is suggested for the determination of riboflavin in the ultraviolet region.

4. The spectrophotometric and polarographic methods require higher concentrations of riboflavin than those required in the fluorometric method. The spectrophotometric method requires 2 to 5 μg./cc. while the polarographic method requires 10 to 100 μg./cc. in contrast to 0.1 to 0.2 μg./cc. required for the fluorometric determination.

5. The results obtained by the polarographic method are in agreement with the previously published results (3, 9). The addition of the unknown to 0.02 *N* acetic acid containing 0.1 *N* potassium chloride before polarographing produced satisfactory results for determining riboflavin in tablets and some liquid preparations.

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The Structure and Constituents of *Kleinia Aizoides* DC.*

By ESTEBAN NUNEZ-MELENDEZ† and CARL H. JOHNSON

The microscopic structure of the leaf, stem, and root is described; it is featured by the presence of oil canals. The fresh leaves contain 95.83 per cent water and the dried leaves have 23.46 per cent ash. Other substances present are choline, fat, phytosterol, inulin, and citric, malic, and oxalic acids. A substance similar to vitamin K_1 is indicated by infrared analysis and activity in chicks.

THE PLANT, *Kleinia aizoides* DC., known by the Spanish name of "Mala," has been used as a folk remedy in Puerto Rico with striking results in external and internal bleeding, especially that due to tuberculosis. Because of the local interest aroused, this investigation was undertaken. In this paper the microscopic structure and the chemistry are described, leaving the pharmacology to a later publication.

Kleinia aizoides has been referred by some authors to the genus *Senecio*, but Bentham and Hooker (1) subdivided the latter into twenty-five genera, including *Kleinia*, the name which is generally applied at present.

Descriptions for the species by De Candolle (2) and Harvey and Sonder (3) do not point out that the leaves and younger stems are quite fleshy, to the extent that the plant is a succulent like the other *Kleinias*.

All the material employed in this study was collected in Puerto Rico and was identified as *Kleinia aizoides* DC. by J. I. Otero, taxonomist, now retired, of the Agricultural Experiment Station, Rio Piedras.

MICROSCOPIC STRUCTURE

The only reference in the literature to the histology of *Kleinia aizoides* is a very brief description of the leaf by Jackson and Thoday (4).

Histological studies were made of young and mature stems, leaves, and roots. The material was killed and fixed with Allen's reagent since the usual formalin-aceto-alcohol fixative caused a marked shrinkage of the fleshy material. The tissues were dehydrated with tertiary butyl alcohol and infiltrated with paraffin. The sections were stained with either the safranin-fast green combination or safranin-Mayer's haematoxylin. Temporary mounts were also examined.

The Leaf.—The leaf (Fig. 1 and 2) of *Kleinia aizoides* DC. is subterete. It possesses a well-defined epidermis of rectangular cells, the outer walls of which are thick and covered with a cuticle while the inner ones are thin. The minute stomata are slightly elevated, with the outer walls of the guard cells somewhat thickened. The stomata lead to intercellular spaces in the palisade parenchyma. Trichomes and other epidermal modifications are absent. The epidermal cells, in surface view, are isodiametric except in the furrows where they are

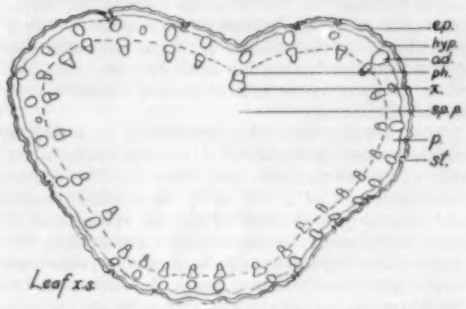

Fig. 1.—Diagram of a cross section of a leaf. ep., epidermis; hyp., hypodermis; o.d., oil duct; ph., phloem; x, xylem; sp.p., spongy parenchyma; p., palisade parenchyma; st., stoma. (×10).

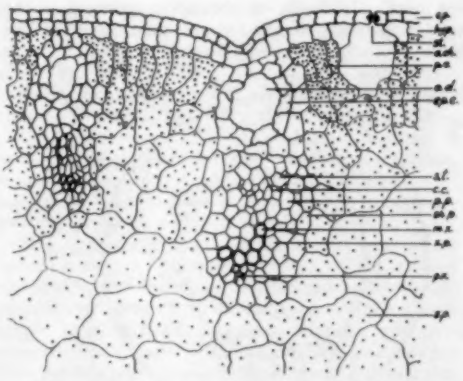

Fig. 2.—Cross section of a portion of a leaf. ep., epidermis; hyp., hypodermis; st., stoma; a.ch., air chamber; p.c., palisade cell; o.d., oil duct; ep.c., epithelial cell; s.t., sieve tube; c.c., companion cell; p.p., phloem parenchyma; sh.p., sheath parenchyma; m.x., metaxylem; x.p., xylem parenchyma; p.x., protoxylem; s.p., spongy parenchyma. (×135).

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elongated. The stomata are of the Ranunculaceous type in which the cells surrounding each stoma are similar to the other epidermal cells.

Just beneath the epidermis is a single layer of hypodermis composed of rectangular nonlignified cells, larger than those of the epidermis. Below the hypodermis are two to four layers of palisade parenchyma. Chloroplasts are mostly concentrated in this zone.

Oil ducts occur in the upper part of the palisade zone and adjacent to the hypodermis. These structures have a well-defined fragile epithelial lining. It may be noted that these canals lie between the larger vascular bundles and the hypodermis, forming radial zones nearly free of chloroplasts. These zones interrupt the palisade layer and are usually indicated on the surface by small furrows, with the effect that the leaves exhibit parallel venation with no definite midrib.

The vascular tissues are distributed in a ring of widely spaced open collateral bundles immediately central to the palisade parenchyma. The largest bundles consist of a few large, thick-walled, spiral and annular vessels making up the xylem, and of sieve tubes with companion cells comprising the larger phloem area, while the other bundles, which are only slightly smaller, have much the same pattern. No fibers are present. The entire bundle is surrounded by a sheath of large parenchyma cells which are elongated with the long axis of the leaf and are practically free of chloroplasts.

The central and major portion of the leaf consists entirely of polygonal and mostly isodiametric parenchyma. Unidentified micro-crystals are present here. The cells of the upper portion of the spongy parenchyma are larger than those of the lower.

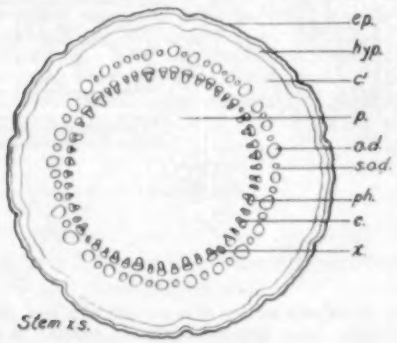

Fig. 3.—Diagram of a cross section of a stem. ep., epidermis; hyp., hypodermis; c'., cortex; p., pith; o.d., oil duct; s.o.d., small oil duct; ph., phloem; c., cambium; x., xylem. (×9).

The Stem.—The stem (Fig. 3) of this plant is subcylindrical. Its epidermis has the same pattern as that of the leaf, but with fewer and smaller stomata and substomatal chambers. There is also a layer of subrectangular hypodermal cells with somewhat thickened walls. Adjacent to the hypodermis is a zone of two to five layers of collenchyma. The major portion of the cortex is parenchymatous. Very few chloroplasts are present, in spite of the external green color of the stem. Small clusters of unidentified micro-crystalline prisms can be observed in temporary mounts of the cortex and pith.

Among the innermost cortical cells and external to the vascular bundles is a circle of oil ducts, each lined with an epithelial layer. They are more deeply situated in the stem than in the leaf.

The vascular bundles are distributed symmetrically in the stele. In the young stems the vascular bundles consist of a protoxylem of spiral and annular vessels and tracheids, and also a protophloem of rather small sieve tubes with companion cells and phloem parenchyma, together with an intermediate very narrow zone of cambium. The sheath of parenchyma enclosing each bundle is much less conspicuous than in the leaf. Small patches of undifferentiated mother phloem cells are scattered between the complete vascular bundles.

The central portion of the stem consists of numerous thin-walled parenchyma cells that constitute the pith.

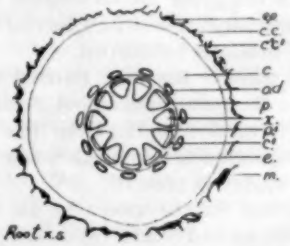

Fig. 4.—Diagram of a cross section of a root. ep., epidermis; c.c., cork cells; c'.c'., cork cambium; c., cortex; o.d., oil duct; p., phloem; x., xylem; p'., pith; c'., cambium; e., endodermis; m., mycorrhiza. (×12).

Older stems differ from the younger in two features, one in which several layers of cork replace the epidermis. Frequently it appears that the hypodermal layer is actively dividing and at other times the epidermis instead seems to have given rise to phellogen. The other difference involves the greater maturity of the vascular tissues. The metaxylem is increased in amount with the protoxylem remaining in the inner portion. The metaxylem consists of a few vessels, together with numerous tracheids and some parenchyma. Fibers are few or completely lacking. The cambium and metaphloem are typical while the protophloem is a crushed mass of cells. Between the bundles are medullary rays of rectangular cells traversed by the interfascicular cambium.

The Root.—The root (Fig. 4), often bearing mycorrhizae, soon loses its definite epidermal cells, which are replaced by cork cells. Cork cambial cells can be observed in this periderm layer. An abundant cortical parenchyma of thin-walled cells contacts this outer zone of corky tissues.

The root is a polyarch siphonostele. The xylem area appears more or less stellate in the transverse section of the root. The larger areas of the metaxylem can be distinguished readily by the thick-walled, strongly lignified angular cells. Some xylem parenchyma is present. In the younger roots and located between the inner tips of the xylem areas, are small patches of protoxylem elements consisting of smaller thick-walled lignified cells,

surrounded by parenchyma. The cells forming the protophloem cannot be distinguished sharply as this region appeared crushed. Some sieve tubes of the metaphloem can be seen interspersed with companion cells. Between the xylem and phloem areas, the cambium can be observed.

The endodermis forms a layer outside of the stele consisting of mostly oval to elliptical cells. The cell walls are thin and the Casparian strips can be seen across the face of many of these cells; Casparian dots are present on the transverse walls of others. Just inside of the endodermal layer is a row of polygonal and thin-walled cells of the pericycle. In older roots the endodermis and pericycle are indistinct.

The large oil ducts occur in the cortex, abutting the endodermis and opposite the large arches of the metaxylem, and are interceded by smaller canals. The ducts attain the size of the largest ones in the stems and leaves. In most cases the epithelial lining is so fragile that it is collapsed in the preparation of the slides.

The central portion of the root is composed of pith, consisting of thin-walled nearly isodiametric parenchyma.

CHEMISTRY

The material used for chemical analysis was first partially dried in the sun, or in an oven at 50°, to prevent rapid decomposition. The water content by this means averaged 95.56%. Further drying by the toluene distillation method of the U. S. P. (5) showed 6.0% moisture in the dried sample, or 95.83% on the fresh basis. The oven drying method showed that the total volatile material of the dried sample was 7.26%.

The total ash of the dried leaves by the U. S. P. method (5) was 23.46% and the acid-insoluble ash 0.62%.

The acid-soluble constitutents of the ash from the leaves were identified by the methods of Treadwell and Hall (6) and of Barber and Taylor (7). Phosphate was precipitated as ammonium phosphomolybdate. Zinc formed the white zinc sulfide. Iron reacted with potassium ferrocyanide and with ammonium thiocyanate. Aluminum gave a positive test with the aluminon reagent. Calcium was precipitated as the sulfate, magnesium as the ammonium phosphate compound, potassium as the cobaltinitrite and sodium as the zinc uranyl acetate.

Leaf sections were tested for alkaloids by the iodine-potassium iodide method described in Manske and Holmes (8). A brownish-red precipitate was very abundant in the outer palisade cells, the epidermis, and the outer parenchyma of the pith; in the latter it was concentrated near the cell walls. The precipitate was not due to protein, as indicated by treatment of other sections with tartaric acid solution.

Other histochemical tests described by Johansen (9) were carried out on fresh leaves. Sudan III gave the orange-red color characteristic of fats in the oil tubes and in globules near the periphery of the sections. Concentrated sulfuric acid produced red masses, eventually becoming olive-green, in the oil tubes and vascular bundles, indicating the presence of phytosterol. Upon dehydration with absolute alcohol the parenchyma around the bundles showed large star-shaped forms and numerous sphaero-crystals of inulin; these could be stained with orange G. Tunmann and Rosenthaler's procedure (10) for plant acids in small pieces of tissue gave positive reactions for citric, malic and oxalic acids, the latter one previously reported by Jackson and Thoday (4). No tannins, saponins, or reducing sugars were found in the sections by Johansen's methods.

Plant extractives.—The percentage extractives obtained with successive solvents were as follows: Petroleum ether, 8.50%; ether, 2.79%; chloroform, 1.15%; alcohol, 0.26%; water, 32.60%.

A portion of the petroleum ether extract was redissolved in the same solvent and shaken with acidulated water. After standing and filtering, the water gave precipitates with phosphotungstic acid, iodine-potassium iodide, mercuric chloride, and Reinecke salt solutions, indicating alkaloid-like constituents. Hydrolysis and subsequent testing for reducing sugars with Benedict's solution showed that glycosides were probably absent. By the method of the U. S. P. (5), the extract was found to contain 20.25% of unsaponifiable matter.

The ether extract yielded very little precipitate with alkaloidal reagents. After this extract was treated with water, acid water, alcohol, and ammonia solutions successively, the insoluble residue melted at 66–69° and gave positive reactions for sterols with Salkowski's and formaldehyde sulfuric acid reagents as described by Klein (11).

The chloroform extract, upon treatment with acid water, yielded very slight precipitates with the alkaloidal reagents phosphotungstic acid, iodine-potassium iodide and mercuric chloride.

The hygroscopic alcohol extract contained no sugars that would reduce Benedict's solution, even after hydrolysis. Tests for tannin with ferric chloride solution and for phenols with Millon's reagent were negative. By the methods in Klein (12) and Robinson (13) tests with phosphotungstic acid, iodine-potassium iodide, mercuric chloride, Reinecke salt, and platinum chloride yielded crystalline precipitates similar in microscopic appearance to those given by a known sample of choline.

The aqueous extract contained no tannins or reducing sugars. A small amount of the extract was dissolved and allowed to stand until a crystalline precipitate formed. Upon carrying out the microscopic silver nitrate, magnesia mixture, and ammonium molybdate tests of Chamot and Mason (14) on this precipitate, characteristic crystals due to the phosphate radical were formed. No sulfates, oxalates, or citrates were present. Upon dissolving the crystals in very dilute acetic acid and reprecipitating with ammonal solution, there appeared crystals microscopicaliy similar to those yielded by known magnesium phosphate.

Since this plant is used to arrest the flow of blood and the clotting time is a factor in this, a test for possible vitamin K_1 activity was carried out qualitatively by the procedure of the A.O.A.C. (15). Groups of day-old chicks were fed the diet suggested by Quick and Stefani (16) until depleted of vitamin K_1 as shown by a prothrombin time of over fifteen minutes. They were then fed dried leaf material of *Kleinia aizoides* in their diet and the prothrombin time determined by the Quick (17) method. The prothrombin time of none of the eight chicks on a

vitamin K_1-free diet was less than three minutes, whereas for those receiving 10 gammas of menadione daily the average time was seventy-five seconds, and for those eight chicks on a diet containing 1% of dried leaves the average time was one hundred and five seconds. The dried leafy material was merely incorporated in the diet and therefore the administration, as well as the results, cannot be considered quantitative. When the petroleum ether extract was fed the prothrombin times were inconsistent but indicated some activity.

Infrared Analysis.—As a consequence of the chick experiments an attempt was made to isolate vitamin K_1. The chromatographic method of Williams (18) was used, in which the petroleum ether extract was passed through a column of Decalso,® the eluate through Permutit,® and the eluate from the latter through Darco.® The final eluate yielded two drops of a light yellow oil.

In a Model 21 double beam Perkin-Elmer spectrophotometer with sodium chloride optics, the oil yielded an infrared spectrum similar to those of vitamin K_1 and menadione, except that the three medium bands 6.14, 6.23 and 7.52 microns were absent.

DISCUSSION

The presence of a large amount of mineral matter, as indicated by the high ash content, is in accordance with the results of Chapman (19) and other workers on succulent plants. They established a relationship of succulence to the quantity of inorganic salts.

The presence of an alkaloid-like substance was demonstrated by the positive reactions obtained with the alkaloidal reagents on the sections of leaves and on the petroleum ether and alcohol extracts. This alkaloid-like substance is believed to be choline from comparisons of the precipitates with those prepared from known choline.

Of the extracts tested, the petroleum ether extract was the only one showing a vitamin K_1 activity on chicks. This was expected, since the vitamin is usually associated with the unsaponifiable matter. Vitamin K_1 was not identified as such, since the infrared spectra did not coincide; however, since the spectra were similar, it is believed that a vitamin K_1-like substance is present.

SUMMARY

1. *Kleinia aizoides* DC. is a succulent plant used in Puerto Rico to control bleeding.

2. The microscopic structure of the leaf, stem, and root has been described. Oil canals are present in each outside of the phloem.

3. The total water content of the leaves is 95.83 per cent.

4. The ash of the dried leaves is 23.46 per cent and contains zinc, iron, aluminum, calcium, magnesium, potassium, sodium, and phosphate.

5. Histochemical tests show fat, phytosterol, inulin, and citric, malic, and oxalic acids to be present.

6. The petroleum ether extract contains choline and 20.25 per cent unsaponifiable matter. The ether extract contains sterols. Choline is present in the alcohol extract. Upon standing inorganic phosphate crystals separated from the aqueous extract.

7. The prothrombin time of chicks was shortened definitely by the dried plant and irregularly by the petroleum ether extract. The latter contains a substance similar to vitamin K_1, as indicated by infrared analysis.

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A Taste Panel Study of the Saccharin "Off-Taste"*

By FRED J. HELGREN, MATTHEW J. LYNCH, and F. J. KIRCHMEYER

Standard taste panel techniques have been used to study the incidence of the off-taste of saccharin salt solutions, freshly prepared without heating. Evidence is presented to show that the off-taste of saccharin is intrinsic in the saccharin molecule as tasted by individuals who are sensitive to it. Decomposition products or trace impurities are not essential to the off-taste response. Approximately 25 per cent of the population can be expected to detect the off-taste in saccharin concentrations up to 0.026 per cent, equivalent to about 10 per cent sucrose. It has been shown that the off-taste response was substantially the same for saccharin sodium, saccharin calcium, and for saccharin sodium prepared by new synthetic methods intended to eliminate trace impurities.

SACCHARIN was first prepared in 1879 by Remsen and Fahlberg (1), who noted its marked sweet taste and commented that "the taste is perfectly pure." An off-taste (almond, disagreeable, metallic, bitter, or after-taste) was noted early (2) and often (3–9) in the literature. The off-taste has been described as being the characteristic flavor of saccharin (2), or due to boiling its solution (5), or to excessively high concentration (7, 9). Saccharin has been so important to the every day living of many people on restricted diets that much work has been done to explain and overcome its off-taste. Thermal decomposition has received much attention as a causative factor, with widely varying results and conclusions (5, 8, 10–12). The discrepancy has probably been due to variations in experimental conditions: saccharin vs. sodium saccharin; simple aqueous solution vs. complex mixtures comparable to prepared foods; *p*H; and temperature, such as the boiling point of aqueous solution vs. the higher temperatures of baking.

The degree of decomposition has been judged in various ways, i.e., by taste comparison, by chemical estimation of end products, by physicochemical methods, or by combinations of these methods. The most recent work of de Garmo, *et al.* (13), indicated that saccharin is practically stable at *p*H 3.3 to 8.0 and from 100° to 150°. A later approach is the claim (14) that the off-taste of saccharin is due to trace impurities of intermediates used in the traditional method of synthesis.

The controversy has undoubtedly been heightened by the subjective nature of taste. Whenever taste is involved, there are bound to be differences of opinion, so that taste tests must be made critically. Some of the older workers, notably Pauli (15) and Paul (3), devised good taste panel techniques for comparing sweetening agents, but the critical methods of statistical analysis have not been developed until recent years.

Routine taste panel work, in this laboratory, on the comparison of cyclamate sodium (Sucaryl®[1] Sodium) with saccharin sodium, has indicated that the off-taste of saccharin may be a characteristic of the saccharin molecule for certain saccharin-sensitive individuals rather than being due to the presence of decomposition products or trace impurities. The present work was designed to explore that possibility.

EXPERIMENTAL

Determination of the Relative Incidence of the Saccharin Off-Taste.—Saccharin sodium U. S. P. was dissolved in distilled water, without heating, to make solutions of 0.01%–0.32% concentration. All solutions were freshly prepared and no solution over thirty hours old was used. Any solutions kept overnight were stored in the refrigerator and allowed to come to room temperature before tasting. The tasters, both men and women, were not aware of the composition of the samples nor the purpose of the experiment. The samples, in glass beakers, were presented to the tasters in a random order, and each taster was asked to give his reaction to the sample before tasting the next solution. Only one taster was present in the laboratory at any one time, so that no taster was prejudiced by the replies of others. Fifty-one new tasters[2] sampled the following concentrations of saccharin sodium solutions: 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32%. The results are presented in Table I; six people (11.8%) experienced no off-taste in the concentrations tested.

To check the results given in Table I, twenty-two new tasters sampled saccharin sodium solutions of the following concentrations: 0.015, 0.03, 0.06, 0.12, and 0.24%. The results are presented in Table II; six people (27.3%) experienced no off-taste in the concentrations tested.

The data of Tables I and II are plotted in Figure 1. The least squares regression line, $Y = 56.5x + 1.8$, was fitted to the data where Y is the per

* Received March 24, 1955 from Abbott Laboratories North Chicago, Ill.

[1] Registered trade mark of Abbott Laboratories.

[2] New tasters are individuals who have not previously tasted saccharin in taste panel work.

TABLE I.—NUMBER OF PEOPLE OUT OF 51 TASTERS REPORTING OFF-TASTE AT VARIOUS CONCENTRATIONS OF SACCHARIN SODIUM IN WATER

Concentration, %	0.01	0.02	0.04	0.08	0.16	0.32
People reporting off-taste	0	7	18	27	37	45
Reporting Off-taste, %	0	13.7	35.3	52.9	72.5	88.2

TABLE II.—NUMBER OF PEOPLE OUT OF 22 TASTERS REPORTING OFF-TASTE AT VARIOUS CONCENTRATIONS OF SACCHARIN SODIUM IN WATER

Concentration, %	0.015	0.03	0.06	0.12	0.24
People reporting off-taste	4	6	10	15	16
Reporting off-taste, %	18.2	27.3	45.5	68.2	72.7

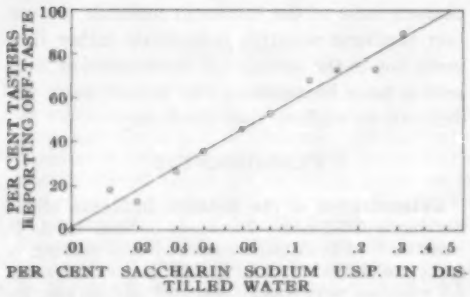

Fig. 1.—Graph of the data presented in Tables I and II showing the least squares regression line.

excellent ($\chi^2 = 8.127$, 9 degrees of freedom, $0.7 > P > 0.5$).[3]

Comparison of the Off-Taste of Various Saccharins.—Nineteen tasters who had been found to be sensitive to the off-taste of saccharin in previous tests compared the off-taste of solutions of three saccharin salts. The solutions were prepared in the same manner as for the first experiment, but the tasters tasted the solutions in order of increasing concentrations, beginning with 0.01% and increasing by 0.01% until the off-taste was noted. The saccharin solutions tasted were saccharin sodium U. S. P. on the first day, saccharin calcium on the second day, and, one week later, saccharin sodium commercially prepared by a new synthesis (14) and claimed to be free of the typical saccharin off-taste. Again the tasters were not aware of the composition of the samples or the purpose of the experiment. Results of this experiment are given in Table III. The analysis of variance of the data is given in Table IV and shows that there is no significant difference between the off-taste threshold of the three compounds tested. The new synthesis has not reduced the incidence of off-taste in saccharin solutions.[4]

CONCLUSIONS

This work supports the indications that the off-taste of saccharin is due to the saccharin molecule itself as tasted by individuals who are sensitive to it. Trace impurities, thermal decomposition or age of solution are not necessary for the appearance of saccharin off-taste. Approximately 25% of the population can be expected to detect the off-taste in saccharin concentrations up to 0.026%, equivalent to about 10% sucrose, and about 50% can detect the off-taste in concentrations up to 0.07%, equivalent to about 28% sucrose.

TABLE III.—MINIMUM CONCENTRATION IN WATER AT WHICH INDIVIDUAL TASTERS REPORTED TYPICAL SACCHARIN OFF-TASTE: IN % X 100

Taster	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Saccharin sodium	3	2	1	1	1	3	4	1	1	1	2	4	3	3	2	1	1	3	7
Saccharin calcium	3	1	2	2	1	1	3	1	1	1	3	1	4	3	2	4	2	2	7
Saccharin sodium[a]	3	2	2	2	1	2	2	1	1	1	2	1	2	3	2	3	1	3	7

[a] Prepared by new synthesis (14).

TABLE IV.—ANALYSIS OF VARIANCE OF DATA PRESENTED IN TABLE III

Source of variation	S.S.	D.F.	M.S.	F ratio
Between tasters	100.38	18	5.577	
Between saccharin salts	.31	2	.155	<1
Error	20.36	36	.566	
Total	121.05	56		

cent people reporting off-taste and x is the log (% concentration × 100 of saccharin sodium). Considering that this is a subjective test, the fit is

SUMMARY

Standard taste panel techniques have been used to study the incidence of the off-taste of saccharin salt solutions, freshly prepared without heating. The percentage of tasters reporting the off-taste over a wide range of concentrations has been determined. It has been shown that the off-taste response was essentially the same for saccharin sodium, saccharin calcium and for saccharin sodium prepared by new synthetic methods intended to eliminate trace impurities.

[3] The authors are indebted to Miss Florence M. Pohley for statistical analysis.

[4] While this paper was in preparation, a reference appeared (16) which apparently covers this point with similar results.

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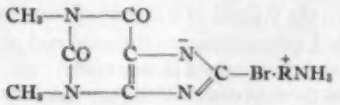
Some New Soluble Salts of 8-Bromotheophylline*

By J. M. HOLBERT, I. W. GROTE, and HARMON SMITH

Over 30 water-soluble salts of 8-bromotheophylline have been prepared and their chemical and physiological properties studied. One of these (the salt 2-methyl-2-amino-1-propanol 8-bromotheophyllinate) has been found to have a low order of toxicity. It has been found to be a useful diuretic in certain types of water retention such as "premenstrual tension" which occurs in many women prior to the onset of menstruation.

PHARMACOLOGICAL and clinical studies involving an 8 - bromotheophylline - antihistamine salt (pyrilamine 8-bromotheophyllinate) revealed the unusual diuretic properties of the 8-haloxanthine portion of the molecule (1, 2). In some respects pyrilamine 8-bromotheophyllinate offered certain disadvantages in clinical work since in the compound the individual dosage of antihistamine and 8-bromotheophylline could not be independently regulated. Therefore, it was decided to investigate the possibility of developing a soluble salt of 8-bromotheophylline that could be used in admixture with an antihistamine salt, thus allowing the dosage of each ingredient to be adjusted at will. Since it is well known that the halotheophyllines are more acidic than theophylline itself, it was decided to prepare a series of 8-bromotheophylline salts of readily available amines in the hope of finding a stable, soluble salt of high diuretic activity. During the investigation over thirty such derivatives of 8-chloro- and 8-bromotheophylline were prepared and screened for diuretic activity; however, those reported in this paper will be limited to the 8-bromotheophylline derivatives which were well characterized.

Although the amine salts of 8-bromotheophylline may be prepared by several modifications, the basic procedure was to react 8-bromotheophylline with an aqueous or alcoholic solution of the amine. Occasionally gentle heat was necessary to effect complete reaction after which the solution was filtered to remove any suspended matter and then evaporated at reduced temperature and pressure until crystallization was evident. After cooling to promote further crystallization, the crystals were collected at the pump and dried in a desiccator. In the case of primary monoamines reacted with an equivalent amount of 8-bromotheophylline, the following general formula for the salt is suggested:



In the case of primary diamines it is possible to produce salts containing either one or two equivalents of 8-bromotheophylline. It is interesting to note that no reaction was obtained with aniline or diethylaniline.

The compounds were characterized chemically by determining their 8-bromotheophylline content, the melting or decomposition point, solubility in water, and *p*H of a saturated, aqueous solution. The 8-bromotheophylline content of the salts was determined by the method given for 8-chlorotheophylline in N. N. R. (3).

The diuretic activity of the compounds was determined by a modification of the antidiuretic test for posterior pituitary as given in the British Pharmacopoeia (4) and is described in detail by Bickers and Woods (1). When rats were given measured doses of water orally and placed in a cage suitable for collecting urine, it was found that approximately 100 per cent of the administered water was collected as urine over a four-hour period. When $^1/_2$ unit of pitressin per rat was given subcutaneously to the animals only around 37 per cent of the administered water was recovered over the same period of time. For our particular purpose this situation provided an adequate background of water storage

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TABLE I.—CHEMICAL AND PHARMACOLOGICAL DATA OF AMINE SALTS OF 8-BROMOTHEOPHYLLINE

Amine	XT[a] Calcd., %	XT Found, %	m. p.	Sol.[b]	pH[c]	D. A.[d]
Methylamine	89.2	87.6	225–250[d]	26	8.4	...
Ethylamine	85.2	82.7	290[d]	35	8.9	97
Diethylamine	77.5	76.5	300[d]	36	10.3	...
n-Butylamine	77.5	77.7	295[d]	30	8.0	87
Cyclohexylamine	72.4	70.7	298[d]	20	9.5	104
Monoethanolamine	80.9	77.6	275[d]	22	8.7	87
Ethylenediamine	81.2	80.0	290[d]	4.1	9.0	118
Propylenediamine	77.8	76.2	215[d]	20	9.1	90
Diethylenetriamine	83.3[e]	80.6	140–160[d]	56	9.8	99
2-Amino-2-methyl-1,3-propanediol	71.2	69.3	300[d]	6.0	7.9	108
2-Amino-2-methyl-1-propanol	67.5	70.6	295[d]	30	8.3	116
2-Amino-4-methylpyridine	70.8	70.7	280[d]	Insol.	...	...
Allylamine	82.0	82.9	295[d]	2.5	7.0	84
Benzylamine	70.8	68.0	78	60	8.0	102

[a] 8-Bromotheophylline. [b] Solubility in grams per 100 cc. [c] Saturated solution. [d] Diuretic Activity. [e] Calculated for 2 moles 8-bromotheophylline per mole amine.

against which to measure diuretic activity. For screening purposes, the compound to be tested was given on the basis of 0.1 Gm. per Kg. of body weight dissolved in water and administered in the amount of 5 cc. per 100 Gm. of body weight. Diuretic activity is expressed as the per cent of volume of urine recovered compared to the volume of water administered.

Table I summarizes the chemical and pharmacological data obtained in this study.

It was thought desirable to compare some of the more promising 8-bromotheophylline salts with their theophylline analogs. Accordingly the corresponding amine salts of theophylline were prepared and tested for diuretic activity by the same procedure. Table II gives the results of these comparisons.

TABLE II.—DIURETIC ACTIVITY OF AMINE SALTS OF THEOPHYLLINE AND 8-BROMOTHEOPHYLLINE

Amine	Diuretic Activity: Theophylline	Diuretic Activity: 8-Bromotheophylline
Benzylamine	73	102
2-Amino-2-methyl-1-propanol	66	116
Ethylenediamine	75	118

In every case it will be noted that the 8-bromotheophylline derivative was appreciably more active than the corresponding theophylline salt.

In a comparative study of the diuretic activity of 2-amino-2-methyl-1-propanol theophyllinate and 2-amino-2-methyl-1-propanol 8-bromotheophyllinate, the dosage level of each compound was systematically increased and the per cent of water recovery determined at each level. Figure 1 gives the results of this comparison. It was found impossible to increase the dosage of the theophylline derivative above the indicated level without introducing considerable toxic symptoms in the test animals.

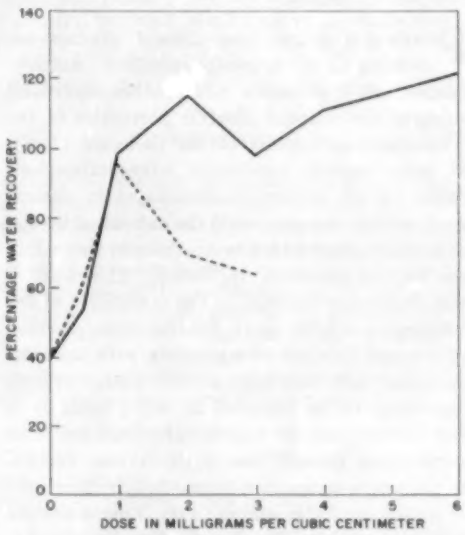

Fig. 1.—Water recovery.

(——) 2-amino-2-methyl-1-propanol 8-bromotheophyllinate.

(----) 2-amino-2-methyl-1-propanol theophyllinate.

The urine samples from the above study were assayed for their sodium, potassium, and chloride content by standard procedures. Figure 2 gives the results from the sodium and potassium determinations while Fig. 3 represents the chlorides.

It will be noted that the maximum response for 2-amino-2-methyl-1-propanol theophyllinate parallels the diuretic response and is reached at a dosage level of 1 mg. per cc., while the maximum

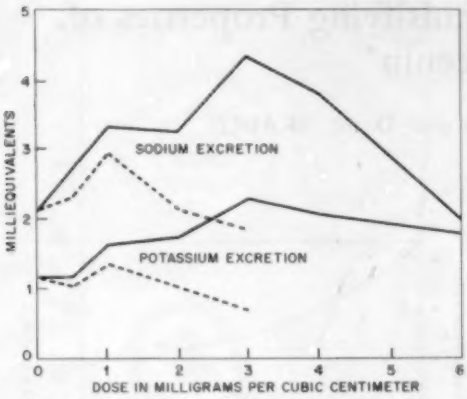

Fig. 2.—Urinary excretion of sodium and potassium.

(———) 2-amino-2-methyl-1-propanol8-bromotheophyllinate.
(- - - -) 2-amino-2-methyl-1-propanol theophyllinate.

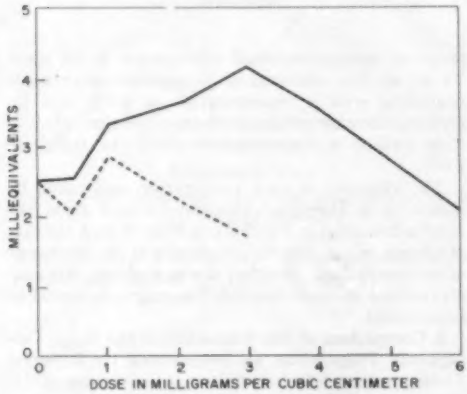

Fig. 3.—Urinary excretion of chlorides.

(———) 2-amino-2-methyl-1-propanol 8-bromotheophyllinate.
(- - - -) 2-amino-2-methyl-1-propanol theophyllinate.

response for 2-amino-2-methyl-1-propanol 8-bromotheophyllinate is reached at a dosage level of 3 mg. per cc.

Because of its physiological activity, high solubility, and low toxicity, one of the compounds in this series, 2-amino-2-methyl-1-propanol 8-bromotheophyllinate, proved to be of sufficient interest to warrant clinical investigation. In combination[1] with pyrilamine maleate it has been found quite valuable in the treatment of premenstrual tension (5, 6).

EXPERIMENTAL

Since the preparation of all compounds reported follow the same general procedure, only a few examples will be given.

[1] Neo Bromth, Brayten.

Methylamine 8-Bromotheophyllinate. — Fifty cubic centimeters of 40% methylamine was diluted with an equal volume of water. 8-Bromotheophylline (25.9 Gm., 0.1 mole) was added in small portions with thorough stirring after each addition. The resulting solution was filtered and the filtrate concentrated at reduced temperature and pressure until crystallization was observed. The concentrate was removed from the distilling flask and cooled to promote separation of the salt. The crystals were removed on a suction filter, sucked as dry as possible, and stored in a vacuum desiccator over calcium chloride for final drying.

Benzylamine 8-Bromotheophyllinate.—To a suspension of 8-bromotheophylline (25.9 Gm., 0.1 mole) in 100 cc. of water was added with vigorous stirring benzylamine (10.7 Gm., 0.1 mole) dissolved in 50 cc. of water. Complete reaction and solution were effected by warming the mixture to approximately 50°. The solution was filtered to remove any suspended matter and evaporated to the point of crystallization at reduced temperature and pressure. In this particular preparation the temperature had to be below 60° to prevent the product from separating as an oil. After cooling, the crystals were collected and sucked dry on a vacuum filter. Final drying was in a vacuum desiccator over calcium chloride.

2-Amino-2-Methyl-1-Propanol 8-Bromotheophyllinate.—8-Bromotheophylline (25.9 Gm., 0.1 mole) was suspended in 125 cc. of water. To this was added 50 cc. of an aqueous solution of 2-amino-2-methyl-1-propanol (8.9 Gm., 0.1 mole). Complete solution resulted on slight warming and vigorous stirring. The solution was filtered and evaporated at reduced temperature and pressure until crystallization of the salt was noted. After cooling the compound was removed by vacuum filtration and dried *in vacuo* over calcium chloride. Results of the analyses for 8-bromotheophylline content are given in Table I.

SUMMARY

1. The preparation and properties of a series of 8-bromotheophyllineamine salts is reported.

2. In general the diuretic activity of the 8-bromotheophylline derivatives appears decidedly superior to the corresponding theophylline derivatives when assayed by the method given.

3. One of these compounds, 2 - amino - 2-methyl-1-propanol 8-bromotheophyllinate, has proved to be of clinical value in the treatment of premenstrual tens'on.

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An Evaluation of the Emulsifying Properties of Carrageenin*

By B. W. FITZGERALD and D. M. SKAUEN

A comparison between various emulsions made with carrageenin and acacia as a standard was made. Individual emulsions were evaluated on the basis of viscosity and particle counts.

CARRAGEENIN[1] is the term most often used today to describe the dried aqueous extract of Irish-moss. The product "Chondrus Extract," National Formulary Ninth Revision, is obtained from one of two species of red algae; *Chondrus crispus* (Linné) or *Gigartina Mamillosa* (Goodenough et Woodward). Since the latest revision of The National Formulary recognized "Chondrus Extract" and because various references (1, 2) to the emulsifying properties of the material appear in the literature, a study was undertaken to determine the efficacy of carrageenin as an emulsifying agent when compared with more widely used agents, particularly acacia.

EXPERIMENTAL

Preservation.—Carrageenin, like other vegetable gums must be properly protected against bacterial and mold growth. Spalton (3) recommends using either 0.2% benzoic acid or a mixture consisting of 0.1–0.2% methyl *p*-hydroxybenzoate and 0.02% propyl *p*-hydroxybenzoate. In this investigation three solutions of carrageenin (1%) were made containing the following: (*a*) no preservative, (*b*) 0.2% benzoic acid and (*c*) a mixture of 0.2% methyl *p*-hydroxybenzoate and 0.02% propyl *p*-hydroxybenzoate. The solutions were observed for one month, and the viscosities of each were measured with a Hoeppler viscosimeter. In that preparation containing no preservative the odor of decomposition and bacterial growth were detected within a few days after preparation. The preparation containing benzoic acid showed some decomposition as shown by a loss of viscosity. Since the preparation containing the methyl and propyl *p*-hydroxybenzoates showed no decomposition as noted by the viscosity changes, Fig. 1, a mixture of the methyl and propyl para esters were used in this study as mold and bacterial inhibitors. In all preparations whether stable or not, no significant change of *p*H was observed.

Relationship Between Concentration and Viscosity.—Prior to the formulation of the emulsions it was necessary to determine a practical working range of concentration of carrageenin to be used. To do so, ten solutions of carrageenin were made beginning with a concentration of 0.2% and increasing the concentration by an increment of 0.2%. This yielded a concentration range of 0.2% to 2.0%.

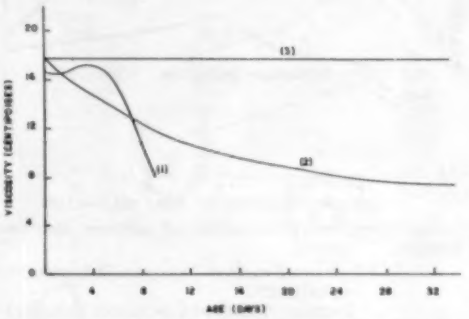

Fig. 1.—Viscosity changes of preserved carrageenin solutions.

The viscosity of each preparation was taken by means of a Hoeppler viscosimeter and from the results described in Fig. 2 it can be seen that the rate of change is not directly proportional to the change of concentration. Further it was realized that concentrations of more than 0.8% carrageenin would be impractical.

A Comparison of the Viscosities of the Same Carrageenin Preparation as Determined by Different Viscosimeters.—It has been previously noted (4) that the viscosities of carrageenin solutions over a wide range of concentrations were not consistent. It was stressed that one must use an instrument operating at a constant rate of shear, and, even then reproducible results will be obtained only in the low concentration range. In the higher concentration ranges thixotropy plays an important role and reliable results will be obtained only when one pays closest attention to amount of agitation and time.

Working with solutions of low concentration of carrageenin (0.2–1.0%) a study was undertaken to establish the best means of making future viscosity measurements. It had been observed while doing some preliminary work that a discrepancy existed when measuring the viscosity of the same solution in the Hoeppler viscosimeter but employing different balls.

Five solutions of carrageenin were prepared by dissolving respectively 0.2, 0.4, 0.6, 0.8, and 1.0 Gm. of carrageenin in 100 cc. of water. The viscosities of these solutions were to be taken by three different methods, (*a*) Saybolt Universal Viscosimeter, (*b*) Hoeppler viscosimeter ball H-8 (metal), and (*c*) Hoeppler viscosimeter ball F-6 (glass). These measurements were conducted at 37.5° since the standard tables in the Pharmacopeia are estab-

* Received August 27, 1954, from the Research Laboratories of the University of Connecticut School of Pharmacy, Storrs.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
[1] The carrageenin used in this study was supplied through the courtesy of the Seaplant Chemical Corporation, New Bedford, Massachusetts.

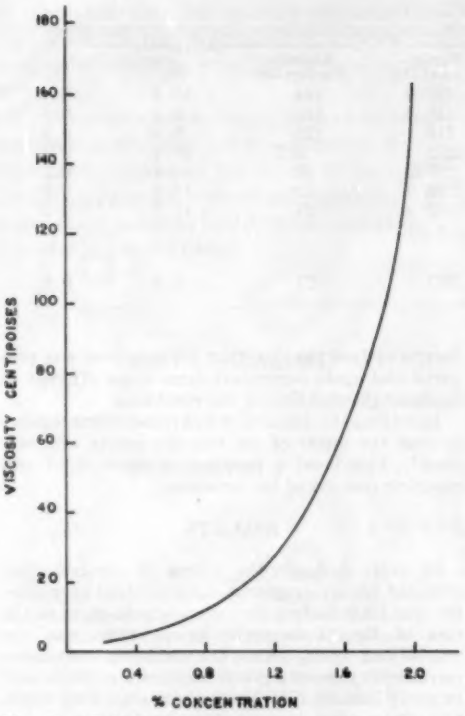

Fig. 2.—Relationship between concentration of solution and viscosity.

lished at this temperature. The results of these experiments will be found in Fig. 3.

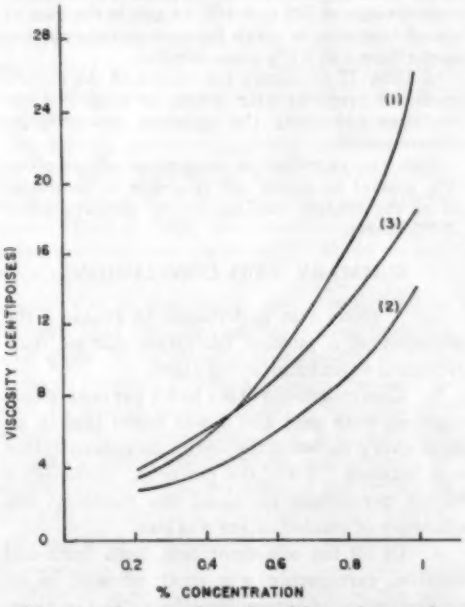

Fig. 3.—Comparative viscosities using various instruments: (1) Hoeppler Viscosimeter Ball F-6; (2) Hoeppler Viscosimeter.

From these results, it was apparent that when determining the viscosity of one set of emulsions with the Hoeppler viscosimeter the same ball would have to be used throughout. If one ball were used to measure the viscosity of the more fluid preparations and another to measure the more viscous ones, there could be no correlation of results.

Since it was difficult to obtain reproducible results with the Saybolt Universal Viscosimeter, this instrument was not used.

Preparation of the Emulsions.—The carrageenin and the water were mixed in a mortar and allowed to stand for one hour to permit complete solution. To this mucilage, the oil was added in small portions and the mixture was triturated. By this method it was possible to suspend the oil in the form of very large globules but, if allowed to stand, the globules of oil coalesced to form a layer. Continued trituration of the oil and mucilage did not produce smaller globules. Therefore, after the oil had been temporarily suspended in the mucilage by trituration in a mortar, the mixture was passed through a hand homogenizer six times. The emulsions produced by this technique were acceptable. The increased emulsification obtained by employing the homogenizer suggested the use of a colloid mill which is, in theory, more efficient and is widely used in large scale operations. Therefore, in this study, both techniques were used to prepare the emulsions and a definite procedure developed for each technic.

In this study a total of seven oils and benzyl benzoate were used to evaluate the efficiency of carrageenin. The oils used and the concentration of the emulsions prepared are shown in Table I:

TABLE I.

Oil	Concentration of Emulsion, %
1. Cod liver	50
2. Liquid petrolatum	50
3. Light liquid petrolatum	50
4. Castor	35
5. Cottonseed	50
6. Linseed	50
7. Turpentine	15
8. Benzyl benzoate	25

Turpentine was chosen to represent a volatile oil and the benzyl benzoate to represent an emulsion in which, according to the official formula, the emulsifying agent is a soap.

Wherever possible, an official formula for the emulsion was prepared as a basis for comparison. In all instances except in the benzyl benzoate lotion and turpentine oil emulsion, 5% alcohol was added as an additional preservative because the *p*-hydroxybenzoates are more readily soluble in the fixed oils, and sufficient preservative may have been partitioned to the oil phase to permit bacterial growth in the aqueous phase.

Analysis of the Emulsions.—**A.** *Viscosity.*—On the day that the emulsions were made and again four weeks later, the viscosity of each emulsion was measured with the Hoeppler viscosimeter (Precision Model HV 303). The emulsions were brought to 25° in a constant temperature bath. Before being placed in the viscosimeter, each preparation was

TABLE II

Emulsion	Acacia		Carrageenin		
	Viscosity (Centipoises)	Particle Count (*H*)	Viscosity (Centipoises)	Particle Count (*H*)	%
Cod liver oil	927	195.9	244	10.1	0.4
Liquid petrolatum	812	145.3	215	2.4	0.4
Light liquid petrolatum	786	216.1	132	0.47	0.3
Turpentine oil	6.59	222.4	9.5	41.4	0.4
Castor oil	146	247.2	39	20.1	0.2
Cottonseed oil	480	226.9	62	16.3	0.2
Linseed oil	115	2.9	21	47.2	0.2
	Soap				
Benzyl benzoate lotion U. S. P. XIV	2.19	333.1	27	2.4	0.6

shaken five times to insure uniformity. The emulsion was then poured into the viscosimeter chamber which was maintained at 25°, and allowed to stand for ten minutes to allow any air bubbles to rise to the surface. Three readings were taken for each sample.

B. *Determination of Globule Size.*—Several methods have been established by which the efficiency of emulsification is judged. The most common method is that of observing the degree to which an emulsion creams. Another method is to express the efficiency of emulsification in terms of the arithmetic mean of the diameters of the globules of oil, (the dispersed phase in this case). This is sometimes useful but is not necessarily the most satisfactory method.

The method used in this study was an adaptation of that described by Smith and Grinling (5) and is as follows: A quantity of diluted emulsion was placed on a Thoma hemacytometer 0.1 mm. deep. Using the ocular micrometer divided into 400 squares of $(0.0175)^2$ mm.^{2} under high power and $(0.07407)^2$ mm.^{2} under low power, the number of globules in 200 squares (40 squares in 5 different fields) were counted. The emulsions were diluted in a medium containing the same percentage of emulsifying agent present in the emulsion and to a concentration such that, when a few drops were placed on the hemacytometer, each square contained about 5 to 20 globules. An ocular micrometer was employed rather than the rulings of the hemacytometer because it was difficult, if not impossible, to observe the oil globules and the markings of the hemacytometer simultaneously.

The degree of emulsification (H) is most conveniently expressed as the number of millions of globules into which one mm.^{3} of oil is subdivided. Thus, the value (H) is independent of the concentration of the emulsion. (H) is the product of the number of globules per square (n), the number of squares necessary to measure 1 mm.^{2}, 10^{-6}, the volume to which 1 cc. of the emulsion was diluted, and the concentration factor of the emulsion.

Thus a particular emulsion, one cc. of which was diluted to 1,000 cc., measured under high power, one square containing (n) number of globules, and containing 30% oil would yield an (H) calculated as follows:

$$H = n \times 32{,}653 \times 10^{-6} \times 1{,}000 \times 100/30$$

If the emulsion were examined under low power, 1,823 would be substituted for 32,653; the remainder of the equation remaining constant. (H) was determined on the day that the emulsion was prepared and again one month later in an attempt to evaluate the stability of the emulsions.

In making the dilutions it was found advantageous to coat the inside of the transfer pipets with silicone.[3] This coating provided a more rapid and complete transfer of the emulsion.

RESULTS

In every instance the degree of emulsification obtained when carrageenin was employed as emulsifier was far less than that when acacia or, as in the case of benzyl benzoate lotion, soap was the emulsifying agent. Also, the emulsions containing carrageenin showed a greater tendency to cream and in many instances the oil in these emulsions began to coalesce several weeks after preparation.

The efficiency of carrageenin decreased when the concentration became sufficiently high to produce very viscous emulsions or when the concentration was too low. In almost every instance, the efficiency of carrageenin reached a peak in the concentration range of 0.2 to 0.4%, except in the case of benzyl benzoate, in which instance carrageenin was most efficient at 0.6% concentration.

In Table II are shown the results of the control emulsions prepared with acacia or soap and the emulsions containing the optimum concentration of carrageenin.

With the exception of turpentine oil emulsion (5% acacia) in castor oil emulsion (9% acacia) all of the control emulsions were prepared using 12.5% acacia.

SUMMARY AND CONCLUSIONS

1. A study was undertaken to evaluate the efficiency of a purified Irish-moss extract (carrageenin) as an emulsifying agent.

2. Concentrations of 0.1 to 0.7 per cent of carrageenin were used and it was found that in almost every instance the optimum concentration was between 0.2 and 0.4 per cent. Although a higher percentage increased the viscosity, the efficiency of emulsification was less.

3. Of all the oils emulsified, both fixed and volatile, carrageenin was most efficient in its emulsification of turpentine oil. In this instance

[3] Desicote, Beckman Instruments Co.

0.4 per cent was the optimal concentration.

4. From the results obtained, it appears that carrageenin should be considered a quasi-emulsifier.

5. Because carrageenin can produce an emulsion when used in very low concentration, it may prove advantageous in the future to study the effect of combining it with other emulsifiers. The desired result would be to reduce the total amount of emulsifying agent used.

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Investigation of the Toxic Principles of *Hippomane Mancinella* L.*

II. Preliminary Isolation of a Toxic Principle of the Fruit

By W. M. LAUTER and P. A. FOOTE

The fresh fruit of *Hippomane Mancinella* L. was found to contain a water-soluble compound very sensitive to oxygen which gave the rubreserine reaction for physostigmine. Alcohol, ether, and petroleum ether extractions of the fruit were undertaken as preliminary steps to determine the toxic properties of the fruit.

THE REVIEW of the toxic manifestations of this plant (1) makes it seem plausible that at least three toxic substances are present in the plant. The fruit was chosen principally as the first step in the investigation because its ingestion frequently had caused death in humans, while physical contact with the bark or leaves had resulted merely in severe skin irritations and impairment of vision.

Ripe fruit, closely resembling crab apples in size and color, was picked from trees near Bear Lake in the Everglades National Park, Homestead, Fla.,[1] in July, 1950. After twenty-four hours the fruit was stored at 5° F. to prevent deterioration.

According to Madaus (2) only a superficial investigation of the latex of the injured bark has been made. The absence of alkaloids, and the probable presence of some glycoside, has been reported. Furthermore, Wehmer (3) remarks that so-called "toxic emanations" of the plant were caused by trimethylamine-like substances. No facts regarding the constituents of the fresh fruit could be found in the literature, hence a systematic method of extraction was undertaken.

* Received August 30, 1954, from the College of Pharmacy, University of Florida, Gainesville.

[1] The authors are indebted to Mr. Daniel Beard, Superintendent of the Everglades National Park, and his staff for their kind cooperation.

The size of the individual pieces of fruit varied from 4.1–7.2 Gm. An average of 100 pieces was 5.9 Gm.

Moisture: 65.1 per cent (after drying at 100° C.).

EXPERIMENTAL

Preliminary alcohol extraction.—Hot alcoholic extraction left 23.5% nonextractable residue. This shows that 11.4% nonaqueous material had been removed by alcohol extraction.

The alcoholic extract was decolorized with petroleum ether, and then evaporated. A residue corresponding to 6.75% of fresh fruit (*Fraction A*) remained.

It was acidic, partially soluble in water, alcohol, and acetone, insoluble in ether, and completely soluble in dilute sodium hydroxide.

Extraction of *Fraction A* with cold water showed that only 2.08% remained undissolved. The dark red aqueous portion was dried and found to give no reaction with mercuric iodide solution, Mayer's reagent, or picric acid. $FeCl_3$ gave a green color reaction.

Chromatography on kaolin and aluminum oxide furnished no definite bands.

After removal of the organic acids with magnesium oxide, the aqueous filtrate was evaporated *in vacuo*. The residue, equivalent to 2.55% of the fresh fruit was found to possess marked toxic effects (4).

Cold Aqueous Extraction.—The preliminary work made it appear reasonable to assume that the fruit contained a rather labile factor, soluble in water. Therefore, an aqueous extract was prepared in the following manner:

250 Gm. of twice-ground frozen fruit was extracted thrice with 250 cc. each of chloroform-saturated distilled water at room temperature.

The combined aqueous extracts were filtered with the aid of 6 Gm. of Hyflo. The clear filtrate was then evaporated *in vacuo* to a volume of 50 cc.

Therefore, 1 cc. was equivalent to 5 Gm. of fresh fruit or roughly to one piece of Manchineel fruit (*Extract C*). Pharmacological tests carried out on this extract are reported by Fox and Ariail (Part III of this investigation).

The residue remaining after aqueous extraction was first air-dried at 45° and then *in vacuo* over sulfuric acid at 30°. It was then extracted with petroleum ether. The green petroleum ether solution was evaporated. Yield of green waxy residue: 1.95 Gm. or 0.78% of the fresh fruit (*Fraction D*).

The water and petroleum ether extracted material was then extracted with ether. After evaporation of the ether, a yield of 0.42 Gm. or 0.17% of fresh fruit was obtained (*Fraction E*). It is a greenish viscous oil and has a mustard-like odor.

The residue remaining after consecutive extractions with water, petroleum ether, and ether was now extracted with hot 95% ethanol. After removal of the alcohol a yield of 2.3 Gm. or 0.92% of the fresh fruit (*Fraction F*) was obtained. This material proved to be a hard black resin.

The material was decolorized by extraction with 25 cc. cold water. A yellowish crystalline residue remained after drying *in vacuo* (*Fraction F-1*).

Yield: 1.02 Gm. or 44.3% of *Fraction F* or 0.41% of fresh fruit.

Fraction F-1 is insoluble in ether, but soluble in warm ethanol from which addition of ether failed to form a precipitate. It is insoluble in water and in 1% hydrochloric acid solution and easily soluble in cold 1% sodium hydroxide solution, but insoluble in cold or hot sodium carbonate solution. This behavior might point to the presence of phenolic groups.

A portion was dissolved in cold 2% NaOH and rapidly assumed a dark red color on standing. An attempt at decolorization with charcoal failed. Dilute hydrochloric acid precipitated a grayish material, which upon filtration turned dark red and lost its ability to dissolve in alcohol.

Apparently alkalinity causes very rapid oxidation to a dark red colored substance. In view of some pharmacological observations on our extracts (see Part III of this investigation), this behavior could be interpreted as an indication that physostigmine or a derivative of it might be present, giving the well-known rubreserine reaction. Since this color reaction is used for assay purposes, it seemed logical to repeat our extraction process using extreme precautions, since this alkaloid is noted for its great sensitivity to oxygen.

This would also explain why earlier investigators had failed to notice an alkaloid of this type since it is unlikely that it would have withstood drying and a lengthy sea-voyage to Europe. One of the decomposition products of physostigmine is methylamine and it might be possible that the "trimethylamine-like toxic emanations" referred to by Wehmer (3) could be caused by such process. We have not been able to detect such "emanations" even after one year's storage at 5° F.

The following method for the extraction of Calabar beans, therefore, seemed the logical choice, properly modified, of course, to fit the physical characteristics of Mancinella fruit.

One hundred and twenty-five grams of frozen fruit was allowed to stand for twenty-four hours in a solution containing 150 cc. absolute ethanol, 15 cc. H_2O, and 0.5 cc. concentrated sulfuric acid. The fruit was then removed, ground twice, and returned to the above-mentioned solution and allowed to macerate for twenty-four hours. After filtration, the residue was further extracted, once with 180 cc. 85% alcohol, acidified with 0.12 cc. concentrated sulfuric acid, and twice with 185 cc. each of non-acidified 85% alcohol. These solutions were combined and 1.5 Gm. sodium bicarbonate added to neutralize part of the acid. In view of the easily oxidizable character of the physostigmine-type alkaloids, a reducing agent was added (0.4 Gm. ascorbic acid +0.4 Gm. sodium ascorbate). This also eliminated remaining free sulfuric acid, but still retained a weakly acid medium. The clear solution was then evaporated at 30° *in vacuo*.

To the viscous alcohol-free residue an excess of sodium bicarbonate was added, and the free alkaloids extracted with five portions of ether. The combined ether fractions were dried with anhydrous sodium sulfate.

Since physostigmine salicylate appears to be especially stable toward oxidation, 0.2 Gm. of salicylic acid in anhydrous ether was added. After standing for twelve hours at −15° a small quantity of fine white crystalline precipitate was formed. After filtration and washing 1.4 mg. (*Fraction G*) was obtained. This material which rapidly assumed the typical pink color of physostigmine-salicylate was dissolved in 2 cc. distilled water for pharmacological testing.

The filtered ether solution which, in addition to an excess of salicylic acid, might contain further active material was dried at room temperature under CO_2. Its aqueous solution (20 cc.) was investigated pharmacologically (*Fraction G-1*).

The original weakly alkaline aqueous syrupy material from which *Fraction G* had been removed by means of ether showed no physiological activity. It appears, therefore, that the modified physostigmine extraction process was successful, although the quantity of material isolated was very small.

The residue remaining after extraction of 125 Gm. of the fresh fruit with acidified alcohol was dried *in vacuo* at room temperature.

Yield: 32.1 Gm. (*Fraction H*) of 25.68% of the fresh fruit. Chloroform extraction of *Fraction H* after removal of the solvent yielded 0.949 Gm. *Fraction H-1* (dark green viscous oil) or 3.25% of *Fraction H* or 0.743% of the fresh fruit. Ash content of *Fraction H* (average of two samples): 3.29% total ash, 0.06% acid-insoluble ash.

COLORIMETRIC PHYSOSTIGMINE ASSAY

The method of Ellis, *et al.* (5), was used and extra safeguards were employed to prevent decomposition of the active principle.

Ninety-six grams freshly ground frozen fruit + 2 Gm. tartaric acid were thoroughly mixed under CO_2 and allowed to stand over $CaCl_2$ in a vacuum desiccator overnight. Loss of weight: 12 Gm.

The material was extracted in a Soxhlet apparatus for two hours with ethanol in a CO_2 atmosphere. The liquid was then removed, and the material extracted with further fresh ethanol. The solvent was removed by vacuum distillation under CO_2. Sodium bicarbonate was added and the material was extracted five times with ether.

The combined ether fractions were dried in the absence of air, and the residue taken up in 20 cc. of water. A suspension resulted which was extracted with petroleum ether until this solvent was colorless. The aqueous suspension was then filtered and stored at low temperature. All operations were carried out under CO_2.

This solution was compared with addition of an internal standard containing 1 mg. physostigmine as salicylate per 1 cc. A Klett-Summerson photoelectric colorimeter was used for measuring the rubreserine reaction.

Nine-tenths cubic centimeters of our unknown solution was equivalent to 0.91 mg. of physostigmine; or 96 Gm. fresh fruit would contain 20.2 mg. physostigmine, or 0.021% using this method.

SUMMARY

A preliminary investigation of the fresh frozen fruit of *Hippomane Mancinella* showed that an acidified alcoholic extraction removed a principle which showed remarkable resemblance to physostigmine. An analytical procedure based upon this alkaloid gave positive results. Further investigation employing larger quantities will clarify this question.

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A New Perfusion Apparatus for the Study of the Effects of Drugs on the Coronary Vessels*

By F. P. LUDUENA, ELLEN MILLER, and W. A. WILT

A new apparatus for the perfusion of the isolated rabbit heart by a modified Langendorff method is described. It utilizes the principle introduced by Katz in methods of perfusion of isolated organs in which the flow is constant and the perfusion pressure changes in parallel with the tonus of the vessels. The apparatus described in this communication includes: a flow meter introduced to measure the rate of inflow in the manometer, a special constant level reservoir fed by an all-glass pump, a mixture of O_2 and CO_2 used to aerate the water column, and an all-glass system, except for the short piece of rubber tubing near the heart, in which the fluid circulates.

NUMEROUS EXPERIMENTAL TECHNIQUES have been used to study the effect of drugs on the coronary circulation. Pharmacodynamic studies designed to determine coronary dilator potency in compounds which may be of clinical value should be carried out in intact laboratory animals under experimental conditions as close as possible to those existing in the clinic. However, methods such as those in which coronary flow is measured in the intact dog or cat or in the heart-lung preparation, valuable as they are for the final preclinical studies, are not suitable for the study of a large number of compounds. Any of the techniques derived from the original Langendorff method (1) in which the isolated heart is perfused through a cannula placed in the aortic stump or in one of the coronaries can be used for this purpose. Drugs can be evaluated on the basis of their effect on coronary flow and rate and amplitude of contractions. Obviously, the knowledge thus gained is incomplete as pointed out by Wegria in his critical analysis of these methods (2). However, in preliminary pharmacological testing of coronary dilators, the purpose is to select by the simplest test available those compounds which do dilate the coronaries, and to eliminate those which do not have such property. In addition, sometimes it is necessary to test compounds which have very weak coronary dilator potency or have other and stronger pharmacological activities in order to establish the structure-activity relationship within the series. Due to low activity or side effects it may not be possible to test these compounds *in vivo*.

Most of the investigators who have employed modifications of the original Langendorff method perfused the heart with one of the saline solutions at *constant pressure* and at body temperature and measured the rate of flow as the indicator of the caliber of the coronary vessels. Gerhard Katz introduced a new general method of perfusion of isolated organs (3) in which the rate of inflow in

* Received November 23, 1954, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

The technical assistance of Mr. Charles Underwood is gratefully acknowledged. We are indebted to Mr. M. Priznar for the drawings.

the perfusion system was constant, but the pressure varied according to the changes in the tonus of vessels of the organ perfused. Perfusion of the isolated rabbit ear was described with the original method. The same technique was used for the isolated ear by other investigators (4–6). Katz and his co-workers later applied this principle of perfusion to the isolated rabbit heart (7). Non-oxygenated Ringer solution was perfused at 38°. In our procedure we have made use of the perfusion apparatus which is described below.

METHOD

An apparatus (Fig. 1) was built in our laboratory for the perfusion of the isolated heart based on the method introduced by Katz (3) but which differs from the apparatus described (7) in various details which will be enumerated later. The perfusion system consists essentially of a constant level reservoir (E, Fig. 2), a flow meter (H, Fig. 3), a calibrated vertical glass tube (J, Fig. 3) in which the perfusion pressure is measured, a constant temperature chamber (*I*, Fig. 3) and an arterial cannula placed into the aortic stump of the heart attached to the outlet (O, Fig. 3) by a short piece of rubber tubing.

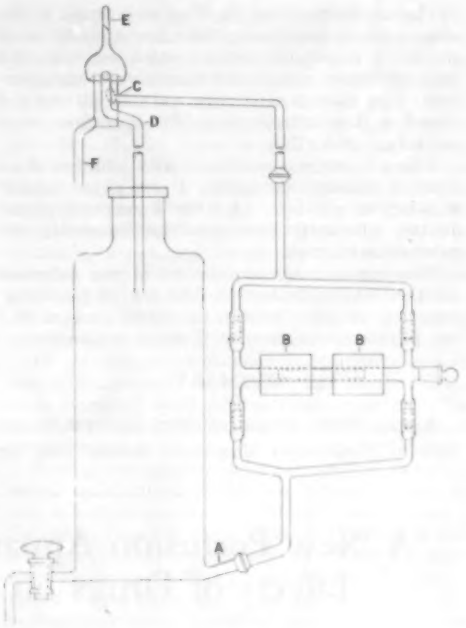

Figure 2.

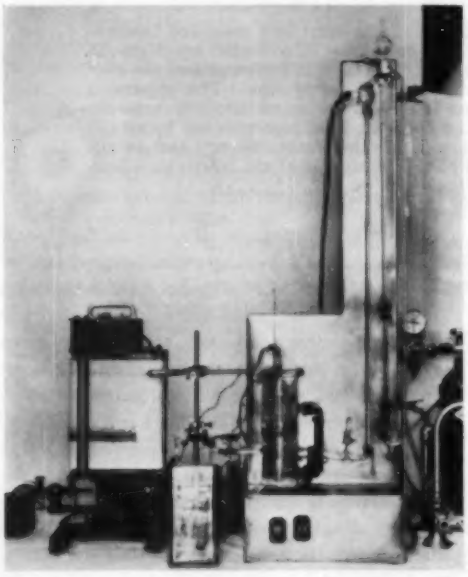

Figure 1.

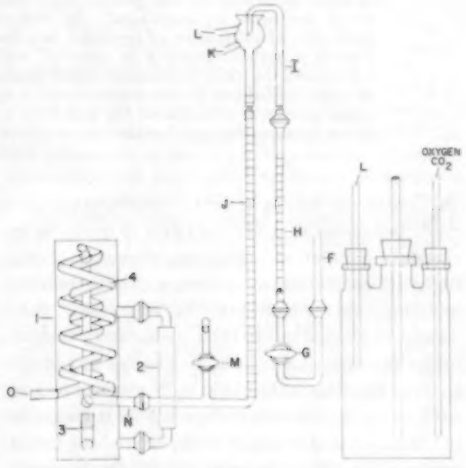

Figure 3.

To begin the experiment, Ringer-Locke solution is poured into the five-liter bottle at the back panel of the apparatus shown in Fig. 2. This low level reservoir is connected by a glass tube (A, Fig. 2) to the pump. On the other side is an outlet which can be opened to empty the bottle. The solution is drawn out of the bottle into an all-glass pump which operates by means of a plunger made of a good grade of magnetic wire enclosed in glass and two solenoids (B, B, Fig. 2). The plunger, in the middle horizontal tube of the pump, is drawn intermittently from one to the other solenoid. The current is supplied intermittently by use of a small motor, not shown in the schematic drawings (Fig. 2 and 3), which has an eccentric shaft. As the eccentric shaft turns, it controls two separate micro-switches which activate the solenoids. The solution is pumped up to the constant level reservoir entering at C at an approximate rate of 100 ml./min. which greatly exceeds the maximum rate of coronary flow in the isolated rabbit heart. A constant level is main-

tained by a return overflow (D) to the original stock solution bottle (Fig. 2) and a vent (E) at the top of the reservoir.

The Ringer-Locke solution flows out at F into a glass column to a pressure type stopcock (G) which is used to adjust the flow through the flow meter (H). From the flow meter the solution flows upward into the column (I, Fig. 3) and drops into a calibrated glass tube (J) engraved with a metric scale from 0 to 75 cm. At the enlarged upper end of the tube (J), there is an outlet (K) for emergency overflow and the opening of a glass tube (L) for the introduction of a gas mixture made up of 95% oxygen and 5% carbon dioxide which has bubbled through water in a Wolff bottle (Fig. 3). The funnel-like opening of the tube (J) is covered loosely with a perforated circular piece of cellophane. From the tube (J) the solution flows to a constant temperature bath (*1*) entering at N. Air bubbles which may form in the tube (J) are eliminated by means of a stopcock (M). The solution flows upward in the center column of the bath, spirals downward through the inner coil and emerges at O. A common T-shaped arterial cannula is attached to the outlet (O) by a short piece of rubber tubing. The heart is attached to the cannula, the tip being placed into the aortic stump. The free branch of the cannula is closed with a piece of rubber tubing and a screw-clamp which can be opened to release air bubbles.

The glass constant temperature bath (*1*) is a compact unit with a second built-in coil (*4*) which may be connected to a cold water faucet to act as a cooling system when room temperature exceeds that required for the experiment. It also has a side-arm type heater (*2*). Air to circulate the water in the bath is introduced through fritted glass (*3*) providing numerous small air bubbles for agitation. An electric thermoregulator sensitive to 0.02° is used to control the heating element. To facilitate dismantling of the apparatus and for easy handling in cleaning, the various pieces are connected by means of joints of the ball-and-socket type held tightly together with the corresponding clamps (the joints without the clamps are shown in the drawings).

The procedure for the perfusion of the heart is as follows: The rabbit is killed with a head blow, the chest is opened, the heart excised rapidly by cutting the large vessels and brought to the cannula, inserting the tip into the aortic stump and held there by a ligature. Stopcock G is turned gradually to provide a flow rate of 5–15 cc./min. The vertical free arm of the cannula is opened and the fluid overflows. The heart is squeezed gently manually; bubbles rise in the cannula and are eliminated with the overflow. This outlet is finally closed and the perfusion fluid flows through the coronary vessels if the left heart valves are not insufficient.

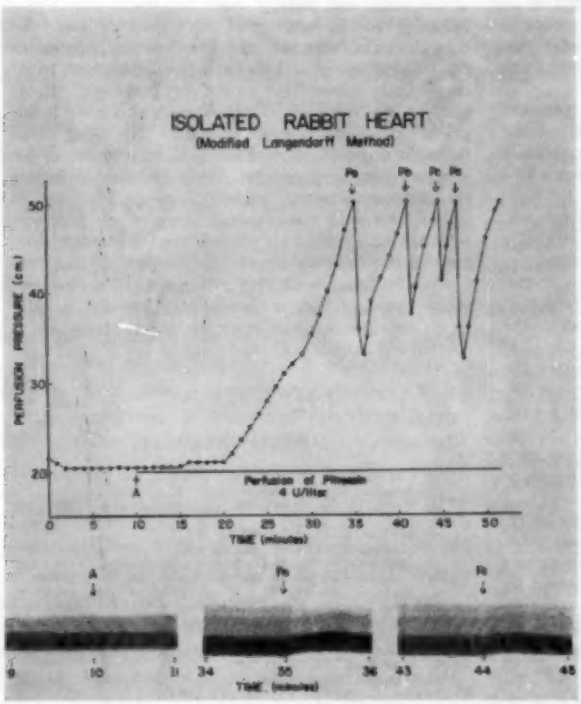

Fig. 4.—Upper tracing: perfusion pressure (cm. water) Lower tracing: myocardiogram. Pa = injection of Papaverine HCl, 50 μg.; Pb = Papaverine HCl, 25 μg.; Pc = Papaverine HCl, 12.5 μg. At *A*, Pitressin® was added to the reservoir, taking a few minutes to reach the heart. The myocardiogram on the left may be considered as a control. The perfusion rate was 7.2 ml./min. during the control period. Due to differences in the time scales, only the effects of two injections (Pa and Pc) are shown on the myocardiogram.

The level of the perfusion fluid rises in the tube (J) until the flow into the tube (J) equals the coronary outflow. In other words, within working limits, the rate of inflow and height of the fluid in the tube (J) will determine the magnitude of the perfusion pressure. If the inflow is constant, changes in the level of the fluid in the tube (J) depend on and indicate changes in the tonus of the coronary vessels. The flow is adjusted to obtain a control perfusion pressure of approximately 20 cm. of water. The reading in the flow meter is recorded at frequent intervals to make sure that the rate of inflow has not changed throughout the experiment.

After starting the perfusion, the level of the fluid column in the tube (J) is read every minute and the values in centimeters are recorded on coordinate paper. If the pressure increases during the control period the inflow is decreased by turning stopcock G. Once an approximately horizontal line of points is obtained, a coronary constrictor is added to the 5-liter reservoir. We have used Pituitrin® or Pitressin® for this almost exclusively in concentrations varying from 1 to 4 pressor units/liter. In a few experiments, naphazoline hydrochloride was used. Both of these drugs have been used for this purpose by the technique described by Ander-

son and Craver (8). The reason for the use of these constrictors is that the coronary arterioles under the artificial conditions of the Langendorff method at 29–30° are almost completely dilated. Only occasionally in hearts not treated with coronary constrictors does moderate dilatation after papaverine or papaverine-like substances indicate the presence of some vascular tonus. A higher degree of coronary tonus was observed when the heart was perfused with Ringer-Locke at 38°. However, the technique described requires a fair degree of constancy in coronary tonus, and for this reason we chose to perfuse the heart at a lower temperature and to rely on a small concentration of Pitressin® to obtain the same effect. Apparently there was no tachyphylaxis inasmuch as the effect of Pitressin® was maintained throughout the experiment (up to four hours).

A few minutes after the addition of the coronary constrictor to the reservoir, presumably as soon as it reaches the vessels, the level of the fluid column begins to rise. The rise continues and if no dilators are added to the perfusion system, the level will exceed 85 cm. in height and reach the overflow outlet (K). When testing coronary dilators, the drug is injected into the short piece of rubber tubing that joins the heart cannula to outlet O as soon as the meniscus reaches the 50-cm. mark. If dilatation occurs the level falls. The amount injected is always 0.1 ml. The changes in perfusion pressure as a result of coronary constriction and dilatation are shown in Fig. 4 which is representative of the effects obtained with Pitressin® and papaverine.

The speed of changes of the perfusion pressure when the inflow remains constant depends on the caliber of the manometer (J) or more accurately on its capacity/unit of length. If the coronary flow ceases, the solution will accumulate in the tube (J) and the magnitude of the rise of the meniscus will depend on the rate of inflow and the capacity of a given length of tube. If partial coronary constriction occurs, only a fraction of the inflow will accumulate in the tube. From this it may be inferred that greater changes can be obtained if the caliber of the manometer is small. However, although the fluid flows along the wall, air bubbles may form in the tubes with a narrow bore. In the apparatus described here, the caliber of the tube (J) is 8 mm. The capacity of the tube has been reduced to approximately 0.2 ml./cm. by the introduction of a glass rod of ±4 mm. in diameter and the same length as the tube (J).

The changes in the perfusion pressure can be recorded kymographically by the use of a glass tube, instead of the glass rod, connected by rubber tubing to a Marey tambour. A cardiogram is obtained by means of a fine hook attached to the apex and connected by thread through a pulley to an ink-writing lever. If the manometric readings are recorded on coordinate paper, they can be transferred later to the cardiogram tracing.

The temperature of the bath is maintained at 29–30°. A series of experiments have been performed at lower (25°) and at higher (38°) temperatures. Rabbit hearts have been used in our experiments. Presumably hearts of larger laboratory animals could be used. This apparatus has been used also to perfuse the rabbit ear for the testing of vasoconstrictors. After the addition of a stable vasoconstrictor, the effect of vasodilators can be tested. It is currently used in this laboratory to perfuse the guinea-pig lung, modified Sollmann and von Oettingen method (9, 10), for the testing of bronchoconstrictors and dilators.

We believe that the isolated heart, as perfused with this apparatus, provides a more sensitive method of testing coronary dilators than the classical Langendorff preparation or those derived from it in which the outflow (or inflow) is measured while the perfusion pressure remains constant. With these methods small changes in flow rate are not usually measured accurately and the experimenter tends to adjust dosages within ranges producing fairly large percentual changes in flow. For instance, if the control flow is 15 cc./min. and following injection of the drug the flow increases to maximal effect of 15.6 cc./min., this would be considered a negligible change and may be even unnoticed if a bucket recorder is used. A similar change in flow in the apparatus hereby described would result in a fall of perfusion pressure of more than 20 mm. Although fairly large and effective doses of papaverine can be injected with little cardiac change, with other compounds chronotropic and/or inotropic effects may be produced with large doses making the interpretation of results more difficult. By using small doses, it is possible to make a large number of injections per preparation. This permits a better comparison between various drugs. The use of large doses with the classical Langendorff preparation can be avoided by placing the very weak solution of the drug in a secondary reservoir and perfusing the heart with this solution until a new and steady level of flow is obtained. However, a relatively small number of tests per preparation can be carried out in this manner.

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The Effects of N-Methylation in 5,5-Disubstituted Derivatives of Barbituric Acid, Hydantoin, and 2,4-Oxazolidinedione*

By THOMAS C. BUTLER

N-methylation of the barbituric acids raises the pK' by about 0.4 unit, greatly decreasing the proportion ionized in the physiological range of pH. Methylation in the 3-position of the hydantoins and oxazolidinediones produces compounds undissociable at physiological pH. Ether/water and benzene/water partition coefficients are increased by methylation. In the barbituric acids methylation raises the apparent volume of distribution (indicative probably of localization in fat), eliminates delay in penetration from blood to brain, decreases renal clearance, and increases anesthetic activity while methylation of the hydantoin derivative, Nirvanol®, decreases anesthetic activity. In the oxazolidinediones methylation of the 5,5-dimethyl derivative produces little change in anesthetic activity while methylation of the 5-ethyl-5-methyl derivative approximately doubles activity. Methylation of barbituric acids confers the properties required of intravenous anesthetics. In clinical use of the methylated antiepileptic drugs it is questionable whether the therapeutic effects are not almost entirely produced by the demethylated products into which the drugs are metabolically converted.

IN PRESENT-DAY medicinal use there are a number of drugs that are derivatives of the closely related heterocyclic structures, barbituric acid, hydantoin, and 2,4-oxazolidinedione, and in which a methyl group has been substituted on nitrogen. Despite the fact that the methyl groups have been introduced into these molecules with the purpose of altering the pharmacological action in a manner favorable for some therapeutic effect, knowledge of the effects of this simple substitution is still far from satisfying. It is accordingly of practical as well as theoretical interest to seek what generalizations can be arrived at concerning the effects of this chemical alteration.

The generalizations to be discussed here are based for the most part on the study of a series which is not extensive but which comprises the compounds of the specified structural types which are in current use as drugs and for which there are the most satisfactory quantitative data relating to physiological disposition. The pairs of methylated and nonmethylated compounds to be considered are listed in Table I. Of these compounds mephobarbital, hexobarbital, Mesantoin®, Nirvanol®, paramethadione, and EMO have asymmetric carbon atoms. Mesantoin®, Nirvanol®, paramethadione, and EMO have been resolved and both isomers of each drug have been studied. Mephobarbital and hexobarbital have not as yet been resolved and only the racemic forms have been studied. It is now known that the methyl group is removed metabolically from all of the methylated compounds in Table I (1–10). In all except hexobarbital the conversion to the demethylated product is almost complete. However, the process of demethylation is sufficiently slow to allow fairly satisfactory pharmacological observations on the unchanged drugs.

PHYSICAL PROPERTIES

The most obvious physical alteration brought about by methylation is in the degree of ionization in the physiological range of pH. As the ionized and nonionized species may be expected to be differently distributed in the body and to act differently pharmacologically, this must be an important factor contributing to pharmacological differences. As is shown in Table II substitution of one nitrogen with a methyl group raises the pK' of the barbituric acids by about 0.4 unit, greatly decreasing the degree of ionization in blood and tissues. In the hydantoins and oxazolidinediones, introduction of a methyl group on the nitrogen in the 3-position eliminates the only proton that can dissociate at physiological values of pH.

Another consistent result of N-methylation is to raise the partition coefficients between various organic solvents and water. As can be seen in Table II the ether/water partition coefficients increase approximately threefold with methylation. In some pairs the difference between the benzene/water coefficients is much greater than this. Fat/water partition coefficients, while of interest in connection with the theories of anesthesia and the distribution of drugs, are more difficult to measure accurately. It is to be expected that the fat/water coefficient will be altered in the same direction as the ether/water and benzene/water coefficients.

VOLUME OF DISTRIBUTION

The apparent volume of distribution of a drug (i. e., the volume in which the total amount of drug in the body would be homogeneously distributed to

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TABLE I.—THE PAIRS OF NONMETHYLATED AND METHYLATED COMPOUNDS DISCUSSED IN THIS PAPER

Nonmethylated	Methylated	References to Metabolic Demethylation
Barbituric Acids		
5,5-Diethyl (barbital)	5,5-Diethyl-1-methyl (metharbital)	(1), (2), (3)
5-Ethyl-5-phenyl (phenobarbital)	5-Ethyl-1-methyl-5-phenyl (mephobarbital)	(1), (4)
5-(1-Cyclohexen-1-yl)-5-methyl ("nor-hexobarbital")	5-(1-Cyclohexen-1-yl)-1,5-dimethyl (hexobarbital)	(5)
Hydantoins		
5-Ethyl-5-phenyl (Nirvanol®)	5-Ethyl-3-methyl-5-phenyl (Mesantoin®)	(6), (7)
2,4-Oxazolidinediones		
5,5-Dimethyl ("DMO")	3,5,5-Trimethyl (trimethadione)	(8), (9)
5-Ethyl-5-methyl ("EMO")	5-Ethyl-3,5-dimethyl (paramethadione)	(10)

TABLE II.—SOME ATTRIBUTES OF THE DRUGS LISTED IN TABLE I

The Compounds Are Arranged in Pairs, the Nonmethylated Preceding the Methylated.

Drug	pK'_a	Per Cent Ionized at pH 7.4	Partition Coefficient[b] Ether/Water	Partition Coefficient[b] Benzene/Water	Volume of Distribution, Per Cent Body Wt.
Barbital	7.8	29	5	0.7	60
Metharbital	8.2	14	18	3	120
Phenobarbital	7.3	56	60	1	75
Mephobarbital	7.7	33	150	>70	200
Nor-hexobarbital	7.9	24	10	0.4	..
Hexobarbital	8.3	11	30	25	..
Nirvanol®	8.5	7	8	0.3	100
Mesantoin®	..	0	26	22	..
DMO	6.2	94	1	0.1	40
Trimethadione	..	0	3	7	..
EMO	6.1	95	5	0.5	40
Paramethadione	..	0	15	50	..

[a] Determined at room temperature by spectrophotometric measurements in buffers of different pH values. The measurements on a pair of compounds were made under the same conditions.

[b] Measurements were made at room temperature. Some coefficients were determined by spectrophotometric measurements on the aqueous phase, some by gravimetric measurements of the residues from evaporation of one or both phases.

give the concentration observed in the plasma) reflects the extent to which the substance penetrates membranes and the extent to which it is localized in tissues. It is an attribute that is determined by the physical properties of the drug, though not necessarily in an obvious manner. In Table II are entered those values for volumes of distribution that have been determined. Most of these values are derived from measurements on dogs, but some have been measured in rats or mice as well. No significant difference between species has been found. Satisfactory analytical methods have not as yet been devised for the determination of some of the drugs in plasma, and the volumes of distribution are consequently unknown. The effect of methylation is seen only in the pairs, metharbital-barbital and mephobarbital-phenobarbital, where both methylated and nonmethylated compounds have been determined. In these pairs methylation is associated with a large increase in the volume of distribution. It appears likely that the high volumes of distribution of the methylated compounds are attributable to localization in fat, and that this localization is the result of high fat/water partition coefficients. Although the volume of distribution of hexobarbital has not been determined, direct analyses of tissues indicate that the concentration is higher in fat than elsewhere (11). The initial rapid fall in plasma concentration after an intravenous injection of metharbital (3), mephobarbital (4), or hexobarbital (11) is due not to chemical alteration of the drug but to passage out of the plasma into the sites of localization, fat being presumably an important site of localization. The transitory nature of the anesthesia produced by an intravenous injection of one of these drugs can be attributed to this physical redistribution within the body, which in turn is determined by the physical properties of the drug.

RATE OF PENETRATION INTO THE BRAIN

Many of the 5,5-disubstituted derivatives of barbituric acid, including barbital, phenobarbital, and nor-hexobarbital, show a delay in the onset of the full anesthetic effects after an intravenous injection (12). This delay is due to slow penetration from the blood into the brain (13). When a barbituric acid derivative having this property is methylated on nitrogen, equilibration between blood and brain becomes rapid and the lag in onset of action after intravenous injection is entirely abolished. The

more rapid penetration into the brain of the methylated compounds appears to be associated with their higher lipid/water partition coefficients. The capillaries of the central nervous system differ from those elsewhere in the body in that the rate of permeation of substances through them is determined by lipid solubility (14).

Nirvanol®, like its barbituric acid analog, has a lag in onset. Its methyl derivative, Mesantoin®, is too insoluble in water to inject intravenously and its rate of penetration into the brain has not been studied. The oxazolidinediones, whether methylated or unmethylated, do not show any delay in onset of anesthesia after intravenous injection. The reason is not apparent why the lag is found with barbituric acids and hydantoins but not with oxazolidinediones.

RENAL CLEARANCE

In all three chemical series, methylation appears to result in a decrease in renal clearance. Simultaneous measurements of the clearances of phenobarbital and mephobarbital in the dog have shown the clearance of the nonmethylated compound to be much the higher (4). Direct determinations of the clearances of the other methylated drugs under discussion are lacking. However, it is known that all of the methylated compounds, even though they may persist in the plasma for considerable times, appear in the urine in no more than insignificant traces. Their clearances must accordingly be very low. It may be that the decrease in renal clearance accompanying methylation is also to be accounted for on the basis of changes in physical properties. It has been suggested (15) that the rate of back diffusion in the renal tubules is determined by the lipid/water partition coefficient.

PHARMACOLOGICAL ACTIVITY

All of the drugs listed in Table I, both methylated and nonmethylated, are capable of bringing about a state of general anesthesia in laboratory animals. The various drugs produce anesthetic effects which, although not qualitatively altogether identical, are superficially so similar as to permit quantitative comparison of activities. In the barbituric acid series, all the methylated compounds, injected intravenously, anesthetize in lower dosage than do their nonmethylated homologs. For those pairs in which both drugs have been determined in plasma (metharbital-barbital and mephobarbital-phenobarbital) the methylated compound has been found also to anesthetize at the lower plasma concentration (3, 4). Valid comparison of Nirvanol® and Mesantoin® is difficult owing to the low water solubility of Mesantoin®, but it does seem clear that the levorotatory isomer of Nirvanol® is actually more potent on the basis of dosage than is its methyl derivative (16). There is no obvious theoretical explanation to account for the effect of methylation in these hydantoins being opposite from that in the barbituric acids. In the oxazolidinedione series methylation of DMO to produce trimethadione is associated with no great change in the dose required to anesthetize mice. On the other hand, either optical isomer of paramethadione is about twice as potent an anesthetic for mice as is either isomer of the nonmethylated homolog, EMO (17).

With the exception of hexobarbital the methylated drugs that are being considered here find their principal application not as anesthetics but as antiepileptic agents. Unfortunately antiepileptic activity is not as readily studied in the laboratory as is anesthetic activity. A number of laboratory procedures have been devised with the view of producing experimental counterparts of the various types of clinical seizures. Tests with these methods have led to the clinical trial of some drugs that have proved to be valuable therapeutic agents. This, however, is not to be taken as an indication that there is as yet any laboratory test that can be relied upon to disclose activity in any close correlation with that in a particular type of human seizure. In laboratory studies of anticonvulsant activity of the methylated barbituric acids, hydantoins, and oxazolidinediones, the tests have generally been made at times after dosage at which the drug would be partially demethylated, but the proportions of methylated and demethylated compounds present in the body at the time of testing have not been known. Testing has not generally been carried out with chronic dosage schedules similar to those used clinically, schedules which result in a much higher proportion of demethylated product relative to unchanged drug than is found shortly after a single dose of a methylated compound. Reports of differences between methylated and nonmethylated drugs as observed in anticonvulsant tests in laboratory animals are accordingly of questionable significance both from the theoretical and the practical point of view.

PRACTICAL THERAPEUTIC CONSIDERATIONS

Aside from matters of theoretical interest there is the important practical question regarding the properties of actual therapeutic value that may be conferred by methylation. If an N-methyl barbituric acid is to be used as an intravenous anesthetic, as is hexobarbital, the characteristic properties associated with methylation, *viz.*, rapid onset and brief duration of action after intravenous injection, are precisely those properties that make the drug suitable for this application. If, however, the methyl compound is to be used in chronic oral administration as an antiepileptic or sedative, the considerations are quite different. When metharbital, mephobarbital, Mesantoin®, trimethadione, and paramethadione are administered in the chronic schedules actually used clinically, there is in every instance direct or indirect evidence to indicate that the product of demethylation accumulates in the body in an amount much greater than that of the original drug (3, 4, 7, 9, 10). It is conceivable that the small amount of unchanged drug is responsible for a significant part of the therapeutic action, but there is at present no convincing evidence that the effects of any of this group of drugs cannot be accounted for as the action of the metabolic product. Despite the fact that there are statements in the clinical literature to the effect that mephobarbital is more efficacious and produces fewer undesirable effects than phenobarbital and that Mesantoin® is less toxic than Nirvanol®, these claims do not withstand critical scrutiny.

There has as yet been nothing approaching a properly controlled clinical comparison of any of the methylated compounds with its nonmethylated homolog. DMO and EMO have not even been subjected to any sort of clinical trial. From the evidence now available it appears dubious whether any distinctive properties of the methylated drugs are actually being utilized when they are administered by mouth in chronic schedules and whether the methylated drugs offer anything more in practical therapeutics than a devious means of administering the corresponding nonmethylated compounds.

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A Study of Methods of Analysis for Boric Acid*

I. Visual and Potentiometric Titration of Boric Acid

By JOHN J. SCIARRA† and JOSEPH A. ZAPOTOCKY‡

The visual and potentiometric titrations of boric acid are discussed. A comparison of the relative effectiveness of various replacements for glycerin in the official assay is submitted. Carbohydrates, alcohols, and polyoxyethylene derivatives were used to replace glycerin in the visual titration of boric acid. A potentiometric method of assay for boric acid was devised utilizing some of the above-mentioned substances.

SINCE 1905, the U. S. P. monograph (1) on boric acid has specified the use of glycerin in the chemical assay. This assay has proved to be reliable, but since the method depends upon the presence of glycerin, satisfactory substitutes for glycerin should be available to the analyst. It seems desirable, therefore, to modify or to change the official method of analysis in such a way as to permit the use of substitute reagents, since it is clear that the analysis should not depend solely upon the use of glycerin.

Boric acid is a weak acid and its alkali salts are hydrolyzed by water to form sodium hydroxide and boric acid. Consequently when an aqueous solution of the acid is titrated with alkali the solution will show an alkaline reaction before the full amount of sodium hydroxide required to neutralize the acid has been added. In the presence of glycerin, however, boric acid can be readily titrated with alkali hydroxide with a sharp end point (2). It is believed that nonpolar substances, such as glycerin, decrease the ionization of the boric acid resulting in a sharp end point (3).

Many substances, other than glycerin, can be used to decrease the ionization of boric acid; but how the results that would be otained by using such substitutes in the assay would compare with the results obtained by the official method has not been known. Such substances include mannitol, invert sugar, fructose, dextrose, and other similar substances. To compare the results obtained using a number of other substances with the results obtained using glycerin, has been one of the objectives of this investigation. Another purpose of this study has been to develop a new method of analysis. This newer method of analysis is given in Part II of this paper.

EXPERIMENTAL

The results obtained by assaying boric acid by the U. S. P. method of assay (4) were taken as the standard for comparison. Any substance that gave a result of 99.5% or more of H_3BO_3 when substituted for glycerin in the assay of this acid was considered to be a satisfactory replacement for glycerin.

Modification of the Official Assay.—The official assay was modified only by the replacement of glycerin with another substance. In the case of liquid replacements, the liquid replacement was directly mixed with water, as is done with glycerin. For

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solid replacements, the solid was first dissolved in one-half the specified amount of water, and the boric acid was dissolved in the other half of the water. The solutions were mixed. Both liquid and solid replacements were neutralized to phenolphthalein before titration. The substances used were as follows: (*a*) *Liquids:* Invert Sugar, Propylene Glycol, Ethylene Glycol, Liquid Glucose, Tween 20, and Tween 80. (*b*) *Solids:* Mannitol, Dextrose, Fructose, Maltose, Lactose, Starch, and Dextrin.

Invert sugar was prepared according to the method of Gilmour (5).

Potentiometric Titration of Boric Acid.—Potentiometric titrations have been used to study the effects of various substances upon boric acid. While this kind of titration has been used quantitatively to determine the amount of boric acid present in a sample, the method has not been adopted for the assay of this acid. There is no advantage to be gained in its use for substances which can readily be titrated visually. But for titrations in which the end point is not definitely shown by an indicator, or when a colored substance interferes in the detection of the end point, potentiometric titrations should be used. Kolthoff and Furman (6), and Kolthoff and Laitinen (7) have outlined the advantages and the varied uses of potentiometric titrations. The potentiometric titrations conducted in this investigation include a study of the effects of various carbohydrates, polyoxyethylene derivatives, and several solvents on the potentiometric titration of boric acid.

All potentiometric titrations were carried out using a Fisher Titrimeter, fitted with calomel and glass electrodes. The instrument was standardized with a buffer solution of pH 7, and the method of operation was that outlined in "Volumetric Analysis with the Fisher Titrimeter," published by the Fisher Scientific Company.

About 40 Gm. of boric acid was weighed and dissolved in enough water to make 1000 ml. A 50-ml. portion of this solution was assayed by the official method for boric acid and found to contain 1.9746 Gm. of H_3BO_3. For the determinations in which carbohydrates and the polyoxyethylene derivatives were employed 50 ml. of this solution was used for each determination. The carbohydrates and polyoxyethylene derivatives were dissolved in 50 ml. of water, the pH was adjusted to 7 with 1.075 *N* sodium hydroxide, and then added to the boric acid solution. The resulting solution was then titrated potentiometrically with 1.075 *N* sodium hydroxide. The ml. of sodium hydroxide used and the corresponding voltage were recorded. For the determinations which studied the effects of solvents on boric acid, about 2 Gm. of boric acid was accurately weighed and dissolved in the solvent. Propylene glycol, ethylene glycol, ethyl alcohol, invert sugar, isopropyl alcohol, and polyethylene glycol 400 were used for this purpose. The solutions were then titrated potentiometrically.

Boric acid alone, boric acid with glycerin, and boric acid with mannitol, were titrated potentiometrically. The relative effectiveness of each of the other substances was determined by comparison with these. Tables I and II show the relative effect of the replacements for glycerin in the assay of boric acid. The effect of the various substitutes for glycerin in this assay on the dissociation of boric acid is given in Fig. 1.

SUMMARY AND CONCLUSIONS

1. A study was made of the relative effectiveness of carbohydrates, alcohols, and polyoxyethyl-

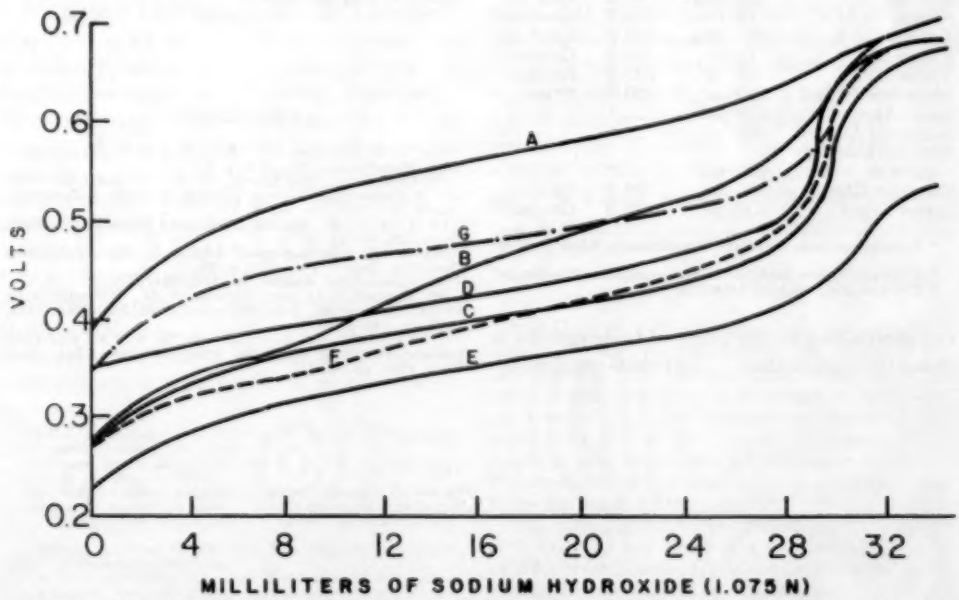

Fig. 1.—Potentiometric titration of boric acid with sodium hydroxide in the presence of various substances. *A:* boric acid alone; *B:* boric acid and mannitol; *C:* boric acid and glycerin; *D:* boric acid and propylene glycol; *E:* boric acid and invert sugar; *F:* boric acid and fructose; *G:* boric acid and dextrose.

TABLE I.—COMPARISON OF THE RELATIVE EFFECT OF CARBOHYDRATES, ALCOHOLS, AND POLYOXYETHYLENE DERIVATIVES ON THE ASSAY OF BORIC ACID BY VISUAL TITRATION METHOD

Substances Used	Amount Used	Boric Acid, %	End Point Change
Glycerin (U. S. P. Method)	100 ml.	99.8	Sharp
Mannitol	10 Gm.	99.9	Sharp
Invert Sugar	100 ml.	99.9	Gradual
Propylene Glycol	100 ml.	99.7	Sharp
Ethylene Glycol	150 ml.	99.4	Gradual
Dextrose	10 Gm.	90.9	Gradual
Liquid Glucose	100 ml.	...[c]	...
Fructose	10 Gm.	99.9	Gradual
Maltose	10 Gm.	50.5	Gradual
Lactose	10 Gm.	57.2	Gradual
Starch	10 Gm.	34.1	Gradual
Dextrin	10 Gm.	...[c]	...
Tween 20[a]	10 ml.	70.7	Gradual
Tween 80[b]	10 ml.	85.0	Gradual

[a] Polyoxyethylene Sorbitan Monolaurate, Atlas Powder Co.
[b] Polyoxyethylene Sorbitan Monooleate, Atlas Powder Co.
[c] The end point was not detected.

TABLE II.—COMPARISON OF THE RELATIVE EFFECT OF CARBOHYDRATES, ALCOHOLS, AND POLYOXYETHYLENE DERIVATIVES ON THE ASSAY OF BORIC ACID BY POTENTIOMETRIC TITRATION METHOD

Substances Used	Amount Used	Boric Acid, %	End Point Change
Boric Acid Alone	....	99.6	Gradual
Mannitol	5 Gm.	99.6	Sharp
Glycerin (U. S. P. Method)	100 ml.	100.0	Sharp
Dextrose	10 Gm.	99.6	Gradual
Fructose	10 Gm.	100.0	Sharp
Maltose	10 Gm.	100.9	Gradual
Lactose	10 Gm.	97.6	Gradual
Starch	10 Gm.	100.9	Gradual
Dextrin	10 Gm.	100.9	Gradual
Tween 20[a]	10 ml.	100.9	Gradual
Tween 80[b]	10 ml.	100.9	Gradual
Propylene Glycol	100 ml.	100.0	Sharp
Ethyl Alcohol	100 ml.	...[c]	...
Isopropyl Alcohol	100 ml.	...[c]	...
Polyethylene Glycol 400	100 ml.	...[c]	...
Ethylene Glycol	150 ml.	99.4	Gradual
Invert Sugar	100 ml.	99.9	Gradual

[a] Polyoxyethylene Sorbitan Monolaurate, Atlas Powder Co.
[b] Polyoxyethylene Sorbitan Monooleate, Atlas Powder Co.
[c] The end point was not detected.

ene derivatives in the assay of boric acid by a visual titration method. The results obtained by utilizing these substances in the U. S. P. assay were compared with the results obtained by using glycerin. Mannitol, invert sugar, fructose, and propylene glycol can be used successfully to replace glycerin in the official visual titration method of assay specified for boric acid. The cost of fructose prohibits its use. Any of the other substances is less expensive than glycerin. When substituted for glycerin, ethylene glycol gives results approaching the official results, but a considerable quantity of ethylene glycol is required. Liquid glucose produced a thick, viscous solution. It is not a practical substitute for glycerin. Maltose, lactose, starch, dextrin, Tween 20, and Tween 80 gave negative results when used to replace glycerin in this assay.

2. Potentiometric titrations were used to assay boric acid. Carbohydrates, alcohols, and polyoxyethylene derivatives were studied and their relative effectiveness in the assay compared. It is possible to assay boric acid by a potentiometric titration utilizing glycerin, propylene glycol, dextrose, fructose, mannitol, or invert sugar. Invert sugar, fructose, mannitol, and propylene glycol gave the best results in the potentiometric titration. Ethyl alcohol, isopropyl alcohol, and polyethylene glycol 400 gave negative results when used in the assay. Polyoxyethylene derivatives were unsuitable. The disaccharides, maltose and lactose, as well as the polysaccharides, starch and dextrin, were ineffective in the assay.

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A Study of Methods of Analysis for Boric Acid*

II. Polarimetric Analysis of Boric Acid

By JOHN J. SCIARRA† and JOSEPH A. ZAPOTOCKY‡

A simple, accurate, and reliable method is desired for the assay of boric acid. A polarimetric analysis has been devised as a solution to this problem. It is easy to determine the angle of rotation of a complex compound formed by boric acid and an optically active substance. The amount of boric acid present can be determined from a standard graph. Polarimetric studies were made of the effect of boric acid on the optical rotation of optically active amino acids, and certain active sugars and hydroxy acids. Tartaric acid and *d*-tartaric acid were found to exert the greatest effect when mixed with boric acid. These two substances are utilized for the polarimetric assay of boric acid.

Boric acid exhibits no optical activity. When boric acid is added to an optically active compound containing hydroxyl groups, a complex compound is formed which has a different optical activity from that of the original optically active compound. With some substances this change in optical activity has been found to be quantitative, with others it is only qualitative. The polarimetric method of assay makes use of those substances which give quantitative results. In such an analysis a standard curve must be made, using known amounts of boric acid and constant amounts of the optically active substance. Once this curve is prepared, it can be used continually to obtain the percentage of boric acid in a solution of unknown concentration.

It has long been known that the addition of boric acid to an optically active compound containing hydroxyl groups will greatly increase the rotatory powers of the compound. Darmois (1) noted the effect of boric acid upon the optical rotation of malic acid. This change in optical rotation is due to the formation of a complex compound of boric acid and malic acid. In 1926, Darmois (2) advanced an explanation for this effect. The complexes formed were of two types: (*a*) boric acid and malic acid formed a levorotatory complex corresponding probably to $H_3BO_3 \cdot 3C_4H_6O_5$, a combination of 1 mole of boric acid with 3 moles of malic acid; (*b*) boric acid and malic acid formed a dextrorotatory complex corresponding to a combination of 4 moles of boric acid and 1 mole of malic acid. The rotatory power of this complex was found to diminish with dilution and temperature changes. With tartaric acid, the complex $H_3BO_3 \cdot 2C_4H_6O_6$, is formed, which is dextrorotatory. In strongly alkaline solution, a levorotatory complex is formed. Optical measurements by Darmois (3) in 1930, showed that the levorotatory complex of boric acid and tartaric acid is unstable in strongly alkaline solutions, probably due to hydrolysis. The presence of neutral salts increase this rotation. Peyroux (4) found, even at saturation, that boric acid is without effect on the rotation of glucose, but that the sodium borates even with the ratio Na:B as low as 1:4 have a large effect. Aurisicchio (5) found that the addition of boric acid does not alter the final value of the rotation of a solution of glucose but that it shortens the time for attaining this value. Tang and Sung (6) found with 5.55 *M* glucose in 1/7.5 *M* boric acid solution the specific angle of rotation to have a sharp minimum of 46.6°. With either greater or lesser amounts of boric acid, the angle of rotation approached the value of 52.5° found for pure glucose solutions.

EXPERIMENTAL

The Kern Full Circle Polarimeter and a sodium vapor lamp were used throughout the course of this investigation. All measurements were made at room temperature since slight changes in room temperature did not show a significant change in the optical activity of the complex measured. Five readings were taken for each substance used, and the average of the 5 readings was recorded. The following method was employed:

The optically active compound (concentrations of 1 to 10%) and boric acid (concentrations of 0 to 3%) were dissolved in enough water to make exactly 100 ml. of solution. A 100-mm. tube and a 200-mm. tube were each filled with the solution. The optical rotation of each was measured. The

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Abstract of a thesis submitted to the Graduate School of Duquesne University by John J. Sciarra in partial fulfillment of the requirements for the degree of Master of Science, June, 1953.
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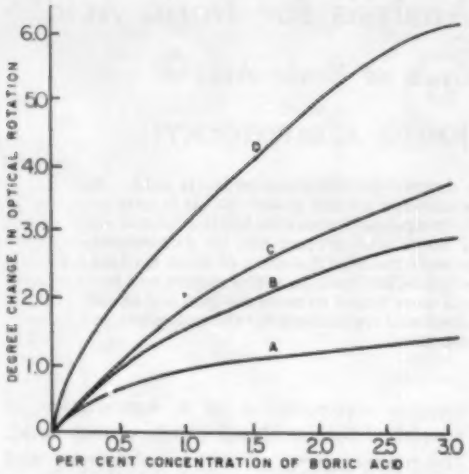

Fig. 1.—Increase in optical rotation of tartaric acid upon the addition of boric acid. *A:* 5% tartaric acid, 100-mm. tube; *B:* 10% tartaric acid, 100-mm. tube; *C:* 5% tartaric acid, 200-mm. tube; *D:* 10% tartaric acid, 200-mm. tube.

optical rotation of the optically active substance alone was measured in a similar manner. For those substances (tartaric acid and *d*-tartaric acid) which formed a complex with boric acid, a graph was plotted showing the relationship between the concentration of boric acid and the increase in the optical rotation. From this graph, the amount of boric acid present in solutions of unknown concentration was determined.

The substances used include: (*a*) *Optically active sugars* (2% Solution): Arabinose, Fructose, *d*-Mannose, and *d*-Xylose. (*b*) *Hydroxy acids:* Citric acid (5% and 10% Solution), Lactic acid (10% and 85% Solution), Tartaric acid U. S. P. and C. P. (1%, 2%, 5%, and 10% Solution), and *d*-Tartaric acid (5% and 10% Solution). (*c*) *Optically active amino acids:* *dl*-Alanine (5% Solution), *l*-Cysteine (5% Solution), Glycine (5% Solution), *l*-Histidine monohydrochloride (2% Solution) and *l*-Leucine (1% Solution).

The optical activity of the amino acid solutions both with and without boric acid was measured within five minutes after the solutions were prepared. Arabinose, mannose, and xylose were treated in a similar manner. The optical activity of tartaric acid and of *d*-tartaric acid was measured after five minutes and after twenty-four hours, and no change in optical activity was observed. Fructose solution was allowed to stand twenty-four hours before any readings were taken in order to allow mutarotation to take place. Boric acid was dissolved directly in the lactic acid solution, and the optical activity measured. Citric acid was treated in a manner similar to the amino acids.

Tartaric acid which is official in the U. S. P. is the dextrorotatory compound and is called tartaric acid. *d*-Tartaric acid is dextrorotatory tartaric acid of the highest purity and is 100% dextrorotatory tartaric acid. Therefore the essential difference between tartaric acid, U. S. P. or C. P. and *d*-tartaric acid is one of degree of purity.

TABLE I.—DETERMINATION OF UNKNOWN AMOUNTS OF BORIC ACID BY POLARIMETRIC METHOD OF ANALYSIS

Tartaric Acid, %	*d*-Tartaric Acid, %	Size of Tube, mm.	Weight of Boric Acid Present, Gm.	Weight Boric Acid Found, Gm.	Boric Acid Found, %
5	..	100	0.9673	0.6000	62.1
5	..	200	0.9673	0.9625	99.5
10	..	100	0.3000	0.2500	83.3
10	..	200	0.3000	0.2700	90.0
10	..	100	0.6000	0.5500	91.6
10	..	200	0.6000	0.6000	100.0
10	..	100	0.8000	0.7750	96.8
10	..	200	0.8000	0.8000	100.0
..	5	100	0.1523	0.1450	95.8
..	5	200	0.1523	0.1520	99.8
..	5	100	0.8450	0.8400	99.4
..	5	200	0.8450	0.8425	99.7
..	10	100	0.1523	0.1500	98.4
..	10	200	0.1523	0.1520	99.8
..	10	100	0.8450	0.8400	99.4
..	10	200	0.8450	0.8450	100.0

Since it was found that tartaric acid and *d*-tartaric acid gave the best results in concentrations of 5% and 10%, solutions of known concentration of boric acid were prepared and the optical rotation of the borotartrate complex plotted on a graph. From these curves the amount of boric acid in a solution was determined. The amount of boric acid obtained from the curve was compared with the amount known to be present. Figures 1 and 2 show the relationship existing between the concentration of boric acid and the increase in optical activity of

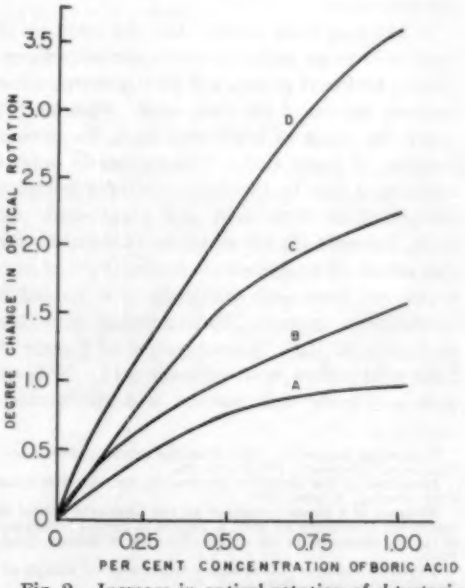

Fig. 2.—Increase in optical rotation of *d*-tartaric acid upon the addition of boric acid. *A:* 5% *d*-tartaric acid, 100-mm. tube; *B:* 10% *d*-tartaric acid, 100-mm. tube; *C:* 5% *d*-tartaric acid, 200-mm. tube; *D:* 10% *d*-tartaric acid, 200-mm. tube.

tartaric acid and *d*-tartaric acid. Table I shows the results of assaying boric acid by this new polarimetric method of assay.

SUMMARY AND CONCLUSIONS

1. The effect of boric acid upon the optical rotation of optically active compounds was studied. Boric acid was found to have no effect upon the optical activity of the amino acids studied. The optical activity of the sugars, arabinose, *d*-mannose, *d*-xylose, and fructose, was only slightly affected by the addition of boric acid. The absence of an increase in optical rotation proved that neither citric nor lactic acid forms a complex with boric acid. Maximum increase in optical activity upon the addition of boric acid was observed with tartaric acid and *d*-tartaric acid, the latter being the more effective. Best results were obtained when the solution was viewed in a 200-mm. tube.

2. A new polarimetric method was devised for assaying boric acid. It utilizes the increase in optical activity to be observed when boric acid is added to solutions of tartaric or *d*-tartaric acids. The best results were obtained when the concentration of tartaric acid or *d*-tartaric acid was 10 per cent, and the concentration of boric acid was 1 per cent or less. The results obtained by assaying boric acid by this new polarimetric method were compared with the results obtained by the standard visual titration method. They were found to be accurate within the limits specified by the U. S. P.

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Thixotropic Measurements of Bentonite Suspensions*

By HARRIS B. BERNSTEIN and MARTIN BARR

The use of the hysteresis loop method for the determination of the thixotropic state of bentonite suspensions has been established and the use of the planimeter to measure hysteresis loops has been introduced. The concepts of the thixotropic unit and the thixotropic ratio were devised so that comparison of the degrees of thixotropy of bentonite suspensions could be easily made. It has been shown that the thixotropic state of bentonite suspensions exists in concentrations as low as 4.3% w/v of bentonite, U. S. P. XIV. The thixotropic behavior of bentonite suspensions was found to be affected by *p*H. Yield values were demonstrated to give an indication of the viscosity of the suspensions.

THIXOTROPY, a reversible sol-gel phenomenon exhibited by many types of colloidal dispersions (1, 2) has not been extensively investigated on pharmaceutical substances. In the measurement of thixotropy, a viscometer must be employed which permits measurement of the shearing stress of a system at different shearing rates. With such a viscometer, one is able to measure the the thixotropic breakdown and buildup of a system and obtain readings which, when plotted, give a hysteresis loop. The viscometer used in this study is the MacMichael viscometer[1] (3–5) with a 2-cm. bob.

The hysteresis loop is used as the basis for determining thixotropy. When the area of such a loop is determined, the degree of thixotropy of different substances or different concentrations of the same substance can be obtained. The greater the area, the greater is the degree of thixotropy (Fig. 1). Upon extrapolation of the thixotropic buildup line of the hysteresis loop to the torque axis, the yield value can be determined (7, 8).

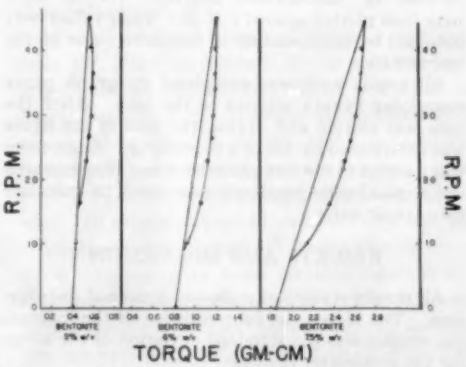

Fig. 1.—Area of hysteresis loop determines degree of thixotropy.

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[1] Gramercy Model No. 15-347, manufactured by Eimer and Amend.

EXPERIMENTAL

Bentonite has been mentioned in the literature as possessing thixotropic properties (1, 5). It was, therefore, decided to employ the hysteresis loop method for the measurement of the degree of thixotropy of bentonite suspensions. Various concentrations of bentonite suspensions were prepared in order to determine the concentrations at which bentonite is thixotropic. The effect of the *p*H on the thixotropy of bentonite was also studied.

The procedure used in preparing the bentonite suspensions was as follows: Into four-ounce graduated (100 cc.) bottles were added 75 cc. of deionized water. The necessary amount of bentonite[1] was then added to each bottle. These preparations were allowed to hydrate for twenty-four hours after each bottle was manually shaken. After this twenty-four hour period, electrolyte (0.5 *N* HCl or 0.5 *N* NaOH) and water were added to the proper volume. Each bottle was again manually shaken and placed into an oven at 30°, for another twenty-four hours. Upon removal from the oven, MacMichael readings and *p*H measurements were made on every suspension.

The concepts of thixotropic unit and thixotropic ratio were devised so that comparison of the degrees of thixotropy could be easily made. In plotting the graphs, the area of 150 squares on the graph paper was equal to 1 r. p. m./Gm./cm. or the product of 1 Gm./cm. times 1 r. p. m. This factor of 1 r. p. m./Gm./cm. is defined as one thixotropic unit. Thixotropic units of the official bentonite magma were given the thixotropic ratio of one. Therefore, subsequent thixotropic substances will be compared with the degree of thixotropy of 5% w/v bentonite magma.

Yield values are expressed in gravitational torque units (Gm./cm.) (6). The absolute torque units are obtained when the gravitational torque units are multiplied by the constant for acceleration due to gravity (980 cm./sec.^{2}).

Three samples for each concentration or *p*H level were prepared and determined. Two determinations were made on each sample and these were averaged.

After the necessary data were obtained, the MacMichael degrees were converted to gravitational torque units by multiplying the wire constant per MacMichael degree by the experimentally obtained number of MacMichael degrees. Torque units were then plotted against r. p. m. Yield values were obtained by extrapolation of the down curve to the torque axis.

All graph work was completed on graph paper containing twenty squares to the inch. After the loop was plotted and drawn, the area of the figure was determined by use of a planimeter. Since there was no need to convert planimeter readings into area units, planimeter readings were used to calculate thixotropic units.

RESULTS AND DISCUSSION

All results statistically showed a normal distribution. The higher the concentration of the magma, the smaller was the standard deviation of the mean for the planimeter readings.

[1] B. C. Volclay Bentonite, American Colloid Company.

Table I shows the average thixotropic ratio for the U. S. P. (5% w/v) bentonite suspensions, selected as the standard for this work. These numbers represent the results of ten determinations made on each (400 cc.) of three separately prepared samples of 5% w/v bentonite suspensions.

TABLE I—AVERAGE VALUES FOR BENTONITE SUSPENSIONS (5% W/V) TO BE USED AS STANDARD

Sample No.	$\overline{Y.V.}$[a]	$\overline{P.R.}$[b]	T.U.[c]	T.R.[d]
1	0.389	15.2	0.675	0.79
2	0.412	23.1	1.028	1.21
3	0.400	19.4	0.862	1.01
Av.	0.400	19.2	0.854	1.00

[a] $\overline{Y.V.}$ = Average Yield Value
[b] $\overline{P.R.}$ = Average Planimeter Reading
[c] T.U. = Thixotropic Units
[d] T.R. = Thixotropic Ratio

TABLE II—THE DETERMINATION OF THE THIXOTROPIC CONCENTRATIONS OF BENTONITE

% w/v Bentonite	$\overline{P.R.}$	T.U.	T.R.
1	...	...	..
2	...	...	..
3	...	...	..
4	...	...	..
4.1	...	...	..
4.2	...	...	..
4.3	13	0.578	0.68
4.4	13	0.578	0.68
4.5	13	0.578	0.68
4.6	13	0.578	0.68
4.7	13	0.578	0.68
4.8	14	0.622	0.73
5.0	19.2	0.854	1.00
6.0	61	2.720	3.19
7.5	139	6.180	7.25

TABLE III—EFFECT OF *p*H ON THIXOTROPY AND YIELD VALUE

% w/v Bentonite	pH	$\overline{Y.V.}$ (Gm./cm.)	$\overline{P.R.}$	T.U.	T.R.
5	2	3.24	...	..	..
	3	2.45	...	..	..
	4	..	...	..	..
	5	..	...	..	..
	6	..	...	..	..
	7	1.10	28	1.2	1.4
	8	0.54	33	1.5	1.8
	9[a]	0.40	19.2	0.85	1.0
	10	0.55	71	3.2	3.7
	11	0.37	65	2.9	3.4
	12	..	...	..	..
7.5	2	4.16	...	..	..
	3	3.91	...	..	..
	4	4.13	...	..	..
	5	..	...	..	..
	6	2.63	94	4.2	5.0
	7	2.93	139	6.2	7.3
	8	2.69	230	10.2	12.0
	8.6[a]	1.80	156	6.9	8.1
	9	2.09	221	10.0	11.7
	10	3.70	430	19.1	22.5
	11	1.55	225	10.0	11.7
	12	..	...	..	..

[a] Represents bentonite suspensions without the addition of electrolyte.

The results obtained in the determination of thixotropic concentrations of bentonite suspensions are shown in Table II. It will be observed that, below concentrations of 4.3% w/v, no planimeter reading is given as no loop was produced. Concentrations above 4.3% w/v exhibit measurable thixotropy. Planimeter readings for concentrations between 4.3% w/v and 4.7% w/v are the same due to the fact that a difference in the slight thixotropic behavior at these concentrations cannot be determined by this method.

Table III demonstrates the effect of hydrogen-ion concentration on the thixotropy and yield values of bentonite suspensions at concentrations of 5% w/v and 7.5% w/v. It can be seen that yield values and loop measurements are independent values, since an increase in one does not necessarily mean an increase in the other. This infers that thixotropic breakdown may be independent of the viscosity of the sample.

This table also shows that, at levels lower than *p*H 8.6, small changes in thixotropy occur but great increases in yield value result. At *p*H levels above 8.6, thixotropy and yield values increase up to *p*H 11, at which a decrease in these values occurs.

Alkaline *p*H produced greater change in thixotropic behavior than acid *p*H; an increase in electrolyte concentration (HCl or NaOH) causes an increase in yield values except at *p*H 11. It was not possible to obtain hysteresis loops for 5% w/v bentonite suspensions at *p*H 2 to 6 and for 7.5% w/v bentonite suspensions at a *p*H of 2 to 5. It was observed that the bentonite was not completely hydrated at these hydrogen-ion concentrations. At *p*H 12, the suspensions were very thick, making it impossible to obtain satisfactory readings with the instrument.

From these results, it may be observed that 7.5% w/v bentonite suspensions are about 8 times more thixotropic than 5% w/v bentonite suspensions when no electrolyte is added.

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A Physical Method for the Determination of Hyoscine Hydrobromide in a Tablet Mixture*

By R. BRUCE SCOTT, ERNEST J. SCHOEB, and J. M. VANDENBELT

A convenient method for the determination of hyoscine hydrobromide in a Benadryl®[1]-hyoscine tablet mixture is described. A first extraction is given the tablet materials with ethanol. This solvent is evaporated and the hydrobromide taken up in dimethylacetamide for measurement of infrared absorption. Although there is approximately one hundred times as much Benadryl® hydrochloride as hyoscine hydrobromide in the formulation, the hydrochloride does not interfere with the assay because of limited solubility in dimethylacetamide and weak absorption at the analytical wavelength.

A METHOD OF ASSAY was needed for the determination of hyoscine hydrobromide in a tablet mixture consisting principally of Benadryl® hydrochloride and milk sugar, with smaller amounts of cornstarch, talc, magnesium stearate, gelatin, and only 0.25 per cent hyoscine. This small quantity of hyoscine hydrobromide present in a Benadryl®-hyoscine ratio of approximately a hundred to one, lends itself poorly to chemical determination.

In an analogous problem, Washburn (1) determined atropine and hyoscine in ointments by conversion of the salts to free bases, which were then taken up in carbon tetrachloride and measured by infrared absorption. The procedure to be described employs a solvent suitably transparent in part of the infrared spectrum, in which the hyoscine salt itself is soluble. The method is thus entirely physical.

The hyoscine and Benadryl® salts are separated from the other ingredients of the mixture by a single ethanol extraction. There is in the infrared spectrum of hyoscine hydrobromide a strong absorption band at 11.61 μ, whereas Benadryl® hydrochloride has a very weak band at 11.58 μ. Although these bands superimpose when the substances are in common solution, the absorption of the Benadryl® hydrochloride under the conditions of the assay remains constant and is of much lower intensity than that of hyoscine hydrobromide. The solvent of choice for spectrophotometric reading was found to be dimethylacetamide: one of its infrared "windows" is in the

* Received December 3, 1954, from the Research Laboratories, Parke, Davis & Co., Detroit 32, Mich.
[1] Parke, Davis & Company registered trademark for diphenhydramine.

11.6 μ region. It readily dissolves the hyoscine salt, but only a limited quantity of Benadryl® hydrochloride.

The two fundamental operations of the assay are: (*a*) extraction of the hyoscine and Benadryl® salts from the mixture with ethanol and subsequent removal of this solvent; (*b*) solution of the hyoscine hydrobromide and part of the Benadryl® hydrochloride in dimethylacetamide for direct measurement in the infrared spectrophotometer.

EXPERIMENTAL

A quantity of the dry mixture comprising 20 crushed (uncoated) tablets, 2.6 Gm., is weighed into a 15-cc. centrifuge tube. Ten cubic centimeters of absolute ethanol is added. Making sure that the powder is dispersed in the alcohol, the tube is shaken vigorously for five minutes. The suspension is centrifuged to give a clear supernatant solution. Without disturbing the solid phase, 8 cc. of the solution is withdrawn with a volumetric pipet and transferred to a 10-cc. Erlenmeyer flask. The alcohol is then evaporated off carefully on the steam bath with the aid of a small air jet. In an hour or so evaporation reaches the point where crystallization of solute begins. At this point the flask is removed from the steam bath and placed in a vacuum oven regulated near 75°. It is important that the alcohol be removed completely, and although four hours in the oven is adequate, it is usually more convenient to continue the treatment overnight.

One cubic centimeter of dry dimethylacetamide previously saturated with sodium chloride is added to the cooled flask, which is stoppered and warmed to 50° for fifteen to thirty minutes. The cake in the bottom of the flask becomes friable during this period; it is broken up with the flat end of a glass rod. After bringing to room temperature, the slurry is poured into a 3-cc. centrifuge tube and spun briefly. A small volume of the clear supernatant is taken off with a dropper to fill a sodium chloride cell of approximately 0.6-mm. path, and is read on the spectrophotometer. The dimethylacetamide used in this determination is saturated with sodium chloride in order to minimize its solvent action on cell walls. Since it is hygroscopic, the flask and centrifuge tube are kept stoppered as much as possible.

In the actual spectrophotometric measurement the spectrum is scanned from 11 to 12 μ against a solvent blank. Figure 1 shows a representative pattern taken directly from a standard run. On the chart a base line is drawn tangent to the absorption minima at 11.02 and 11.82 μ. Transmission readings at the peak and base line of the 11.61 μ band are converted to absorbance values; the difference between the values is the measure of hyoscine hydrobromide content. The assay values are conveniently read off a standard absorbance *versus* concentration line obtained from prepared standards. The equation of this line is $A = 0.091 + 0.0134\ X$, where A is absorbance and X is the number of mg. of hyoscine hydrobromide in the sample equivalent to 20 tablets. The absorption contributed by the Benadryl® hydrochloride does not interfere and is constant in all samples. Since peak intensities are used for the quantitative results, it is advisable that the slit schedule be similar on successive runs (2, 3). All absorption readings in this study were made on a Beckman IR-2T infrared spectrophotometer.

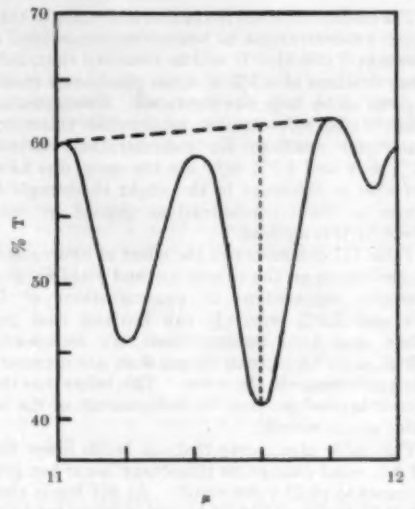

Fig. 1.—The 11.61 μ absorption band of hyoscine hydrobromide in dimethylacetamide.

The recovery of hyoscine hydrobromide from the tablet material by the ethanol extraction was shown to be satisfactory by the following procedure. Weighed quantities of Benadryl® hydrochloride and hyoscine hydrobromide in amounts like those present in assay samples were taken up with dimethylacetamide in the way that regular samples are handled following removal of the ethanol. The absorbance of this solution, saturated with Benadryl® hydrochloride and containing all the hyoscine hydrobromide, was within a few thousandths of that obtained at 11.61 μ in the regular procedure by extraction of the standard amount of tablet material.

RESULTS

Table I gives the values obtained in a number of consecutive determinations over a period of several months. The values are consistently better than 90% of label claims; the mean recovery is 97%. In view of the extremely small quantity of hyoscine hydrobromide in the sample, this must be regarded as acceptable.

The values obtained are for 20 tablets crushed and analyzed before being coated. Several experiments with coated tablets yielded low values, presumably as a result of inadequate extraction. Since dimethylacetamide is hygroscopic, precautions should be taken to prevent gradual addition of water, not only to preserve suitable absorption characteristics but also to prevent attack on the rock salt cell plates. To insure that unfavorable conditions do not develop undetected, it is a useful precaution also to run, with each determination, a standard sample containing a known quantity of hyoscine hydrobromide and appropriate amounts of the formulation ingredients. This serves as a check

TABLE I.—VALUES OBTAINED IN CONSECUTIVE DETERMINATIONS OF HYOSCINE HYDROBROMIDE IN UNCOATED BENADRYL®-HYOSCINE TABLETS

Assay No.	mg./20 Tab. Found	Label Claim, %	Assay No.	mg./20 Tab. Found	Label Claim, %
1	6.05	93	10	6.50	100
2	6.55	101	11	6.55	101
3	6.00	92	12	6.05	93
4	6.50	100	13	6.00	92
5	6.15	95	14	6.00	92
6	6.00	92	15	6.45	99
7	6.55	101	16	6.30	97
8	6.50	100	17	6.30	97
9	6.50	100			

on the extraction and handling procedures, since absorbance of the known sample must correspond with the values obtained for the standard mixtures used in preparation of the absorbance-concentration line.

SUMMARY

An entirely physical method for the determination of hyoscine hydrobromide in Benadryl®-hyoscine tablets has been developed. Better than 90 per cent accuracy is obtained, in spite of an extremely high relative concentration of Benadryl® hydrochloride.

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A Note on a Simple Universal Fluid Drop Counter*

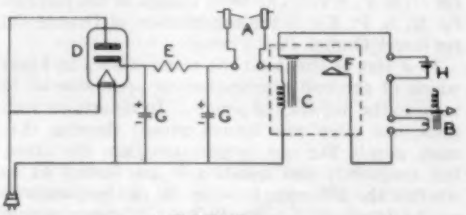
ARTHUR G. ZUPKO and AUBERT CORAN

A simple, easily constructed, economical fluid drop counter is described. Although suitable for most perfusion experiments, it has been found to be particularly efficacious for use with fluids of low electrolyte content such as saliva.

DURING the course of evaluating the antisialogogue activity of anticholinergic agents the need for a suitable device to count and record the drop rate of salivary secretions became apparent.

Many types of drop counters have been described in the literature. One type suggested by Haley and Edwards (1) consists of a transformer which converts the line voltage to eight volts and a "drop gap," through which the drops of fluid pass, each drop making contact and permitting the current from the transformer to flow through a signal magnet. This type, as well as others of a similar nature, is not satisfactory for use with a fluid such as saliva which has too low an electrolyte content. Other types, such as suggested by Winton (2), are sensitive enough to count drops of fluid as low in electrolyte content as saliva, but their construction is too complex and, in some instances, not economically feasible.

A suitable drop counter (Fig. 1) was devised, utilizing a drop gap similar to those previously described. The low electrolyte content of saliva makes its resistance to flow of electric current high, and it was therefore necessary to use a potential higher than that obtained from an eight-volt transformer. Use of a potential as high as 100 volts d. c. resulted in current insufficient to operate the signal magnet, making it necessary to introduce a relay to trip the signal magnet circuit. A diode type vacuum tube was used to convert alternating current from the line receptacle to direct current. A resistor and condenser were used to insure "ripple free" direct current and also prevent damage to the tube or relay in case of a direct short circuit across the drop gap. The parts were mounted on a piece of wood 1 x 5 x 6 in. and wired as shown in Fig. 1.



LEGEND

Fig. 1.—Schematic diagram of drop counter.

A—Drop gap
B—Signal magnet
C—Relay (2500 ohms)
D—Diode vacuum tube (type 117Z3)
E—Resistor (1800 ohms, 10 w.)
F—Lever arm (of relay)
G—Electrolytic condenser (20 mfd)
H—Battery (1.4 v.)

In operation, when a drop of fluid passes through the drop gap, current is permitted to flow into the field coil of the relay, thus creating a magnetic field which forces the lever arm down and closes the signal magnet circuit. The signal magnet, adjusted to write on a revolving kymograph drum, records each drop as an interruption in the continuity of the base line.

Measurement of volume flow may be accomplished by insertion of a graduated container directly below the drop gap. The instrument has proved reliable in recording as many as 60 drops of fluid per minute. The entire apparatus may be constructed for approximately seven dollars.

Over the past two years we have used this simple drop counter for counting and recording salivary secretion from Wharton's duct in dogs and Sten-

* Received August 27, 1954, from the Department of Physiology and Pharmacology, St. Louis College of Pharmacy and Allied Sciences, St. Louis 10, Mo.

son's duct in man. It has also been found to be suitable for use in many other types of perfusion experiments.

SUMMARY

1. A simple, easily constructed, and economical fluid drop counter has been described.

2. Although suitable for many types of perfusion experiments, it has been found to be of particular value for use with fluids of low electrolyte content.

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A Note on the Use of Triangle Design in Taste Testing of Pharmaceutical Preparations*

By MELVIN W. GREEN

IN PHARMACEUTICAL PRODUCT DEVELOPMENT, taste testing is used with considerable frequence both for new products and in modifying formulas for older established products. Statistical design in planning the tests and interpreting the data can be useful in sharpening and judging the results.

In pharmaceutical work the comparison of the taste (or for that matter other aspects also) of two products is made frequently. The triangle design for such tests will be illustrated here using as an example the tasting of Phenobarbital Elixir, U. S. P. XIV and comparing it with the formula as modified for U. S. P. XV. The basic change in the formula for U. S. P. XV is the substitution of Orange Oil for Sweet Orange Peel Tincture.

In a test of this kind the tester wishes to know which of the two preparations is preferable as to taste to the majority of people. In reports on such tests, one often sees figures quoted showing that more people like one preparation than the other, but frequently the question is not asked as to whether the difference between the two preparations can be detected. Obviously if the difference cannot be detected, preference means little. Triangularly designed tasting can be used to determine the ability of the testing panel to differentiate between two preparations.

In triangularly designed tests, the subject is presented with three samples labeled with code numbers or letters. Two of these samples are of the same product. The subject is asked to make two judgments: which sample he prefers and which two samples are alike. This performance is repeated for all tasters. If a statistically significant number of the panel can name the sample pair which are alike, then there appears to be a difference between the two products and one would expect the taster to have a real preference for one or the other.

In the case of the test herein described, the samples were labeled 1, 2, and 3. Samples 1 and 3 were alike. Fourteen tasters were used. By chance alone one would expect one-third of the tasters to select each of the three possible combinations as representing the identical pair, i. e., they would select by chance 1 and 2, 1 and 3, or 2 and 3. Actually 2 tasters selected 1 and 2 (incorrect), 5 selected 2 and 3 (incorrect) and 7 selected 1 and 3 (correct). By chance only one-third of 14 or 4.67 samplers would have made the correct selection. A chi-square test (1) was made to determine whether 7 is significantly different from 4.67, in this case, to indicate that the panel really can differentiate between the products or whether mere chance is operating. Chi-square as calculated by standard methods (1) is 2.71 with two degrees of freedom. Standard tables indicate that chi-square must be equal to or greater than 5.99 to be significant at the 5 per cent level. It can be concluded, therefore, that the tasters cannot differentiate between the products and there is no use looking further into the matter of preference. It is important to realize that the tasters are not selected persons with refined palates but subjects chosen at random.

In setting up a test of this type it is important to present the samples in random order. The following order was used: 3 subjects used the order 1, 2, 3; 1 used 1, 3, 2; 2 used 2, 1, 3; 4 used 2, 3, 1; 1 used 3, 2, 1 and 3 used 3, 1, 2. If there had been a significant preference for one preparation or the other, a very proper question to ask is whether the tasters tend to show preference for a particular preparation by order of taste, i. e., do they select the first or third or middle sample by bias.

To determine this it was shown that 8 of the panel preferred the first sample tasted. The problem now may be posed in such a way that either a taster does or does not prefer the first sample tasted. The significance of the 8 can be determined, then, by running a chi-square test based on one degree of freedom. The test included the usual correction factor for discontinuous numbers (2) and chi-square was calculated to be 0.071. Standard tables entered at one degree of freedom indicated that chi-square must be equal to or greater than 3.84 at the 5 per cent level for significance. This test appears to preclude the likelihood of an undue tendency to select the first elixir tasted. Similar tests for other orders of tasting likewise indicated no bias.

While the data in this paper show that the panel of 14 tasters could not differentiate between the U. S. P. XIV and the U. S. P. XV Phenobarbital Elixirs and that there is no bias in the order of presentation of samples for tasting, the intent of the paper is to show that, by statistical means, taste testing can be made more informative by breaking the test down into components and treating the components statistically. This process is required in designing the experiment as well as in interpreting the findings.

* Received August 27, 1954, from the American Council on Pharmaceutical Education, 77 W. Washington St., Chicago 2, Ill.
The experimental work was done in the laboratories of the School of Pharmacy, University of Wisconsin.

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A Note on a Simple Assay Method for Certain Mercurial Diuretics*

By EDGAR E. THEIMER and PETER ARNOW

The mercury-carbon bond in many mercurial diuretics is rather weak, and the mercury may be converted to titratable form by relatively mild treatment. A simple assay method based on such a titration is described.

THOSE DIURETICS, such as mersalyl, which are formed by the addition of alkyl mercuric salts to compounds containing an allyl group, when dissolved in 50 per cent sulfuric acid, react instantaneously with two moles of potassium thiocyanate, and thus may be titrated just like inorganic mercuric compounds, using ferric alum indicator. Halide ions must be absent. This assay procedure is applicable to injectable solutions of these diuretics, as well as to their organic mercurial components alone. It is a special case of a well-known reaction:

$$RO-\overset{|}{C}-\overset{|}{C}-HgX + HX_1 \rightarrow HgXX_1 + ROH + \overset{|}{C}=\overset{|}{C}\ (X,X_1 = \text{acid radicals})$$

discovered in 1900 (1), and recently used as the basis of a method of determination of unsaturated compounds (2).

METHOD

Weigh or measure accurately a sample containing about 200 mg. of mercury; add 10 ml. distilled water and chill in an ice-bath. Add 10 ml. of sulfuric acid dropwise, with good agitation keeping in an ice-bath ten minutes more. Add 2 ml. of 8% ferric ammonium sulfate and titrate with 0.1 *N* potassium thiocyanate. (Each ml. of 0.1 *N* thiocyanate is equivalent to 10.03 mg. of mercury.)

EXPERIMENTAL

Many assays of various diuretics were run, by the various methods, and typical results are presented in Table I.

Mercumallylic acid and Mercumatilin sodium may give dark decomposition products during titration if treated as directed above. This trouble may be avoided by adding 30 ml. of water instead of 10 ml. Also, in the case of Mercumallylic acid, this powder is best dissolved in about 6 ml. 0.1 *N* sodium hydroxide before addition of the water and sulfuric acid; otherwise it may not dissolve completely.

DISCUSSION

Halide ions interfere in the titration, since they compete with thiocyanate for mercury. A preliminary test for halide should therefore be made. We have found that commercial injectable mercurial diuretics are usually free of halide ions.

Oneto and Cataldi (3) have described a determinative method likewise involving a relatively mild treatment, namely, heating with HCl, HNO_3, and water until colorless. The mercury thus brought into titratable form is treated with standard KCN, and the excess KCN is back-titrated with standard $AgNO_3$ in the presence of ammonia, using KI as indicator. They claim to have obtained precise results for a variety of mercurial diuretics, including mercaptomerin, which cannot be assayed by our method, but they do not mention how long the heating takes.

TABLE I.—MERCURY ASSAYS OF CERTAIN DIURETICS[a]

Mercurial	Hg Found by Proposed Method, Per Cent	Hg Found by U. S. P. or N. N. R. Method, Per Cent	Hg Calculated[c], Per Cent
Mersalyl acid	41.1	41.3	41.5
Mersalyl-theophylline inj. U. S. P.	3.95	4.02	3.9
Acid of mercuri component of mercurophylline	42.4	42.1	41.1
Mercurophylline inj. U. S. P.	3.92	3.92	3.9
Merethoxylline	39.7	39.0	39.3
Merethoxylline-procaine w. theophylline inj. N. N. R.	4.18	4.20	3.93
Mercumallylic acid	40.2[b]	40.3	41.8
Mercumatilin sod. inj. N. N. R.	3.93[b]	3.93	3.9
Meralluride inj. N. N. R.	3.87	3.86	3.9

[a] We wish to thank Eli Lilly and Co., Endo Products Co., and Campbell Pharmaceutical Co. for the compounds supplied by them.
[b] Determined by modified method. See text.
[c] As stated on the label or calculated from the formula.

CONCLUSIONS

In applicable cases, the method proposed herein for assaying mercurial diuretics is fully as reproducible and accurate as the common methods in use heretofore, and has the advantage of being much shorter.

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* Received October 10, 1954, from the Metropolitan Laboratories, Inc., Oyster Bay, N. Y.

A Note on a Modification of the Anderson-Craver Perfusion Apparatus for Interchanging Perfusion Fluids*

By EDWARD B. TRUITT, JR.

THE ANDERSON-CRAVER APPARATUS (1) has provided a convenient design meeting many of the requirements for optimum results during perfusion of insolated hearts as set forth by Chenoweth and Koelle (2). Several modifications have since been described concerning the flowmeter portion (3, 4) and the injection capillary (4).

For the purpose of studying the responses of isolated hearts to known concentrations of drugs, ions, or substrates, the technique of injecting small amounts of a concentrated solution of these substances into the perfusion fluid just above the heart is not satisfactory. No "equilibrium" response is obtained but rather an increasing, then decreasing response. Data obtained from responses to injections of this type are not reproducible because they are somewhat characteristic of the particular apparatus used, and the rate of injection. The effect of removal of ions or substrates from the perfusion fluid cannot be studied. To overcome these difficulties and in particular to adapt the apparatus for the interchanging of perfusion fluids of known composition, a modification of the Anderson-Craver apparatus has been designed in this laboratory and made available by the commercial manufacturer[1] of the instrument. The essential points of the change are shown in Fig. 1. They involve the insertion of a 3-way stopcock (such as Corning No. 7380 with a 2-mm. bore tubing) in the perfusion fluid delivery tube just above the heart cannula. The plug of this stopcock extends to the outside of the warming jacket. Two-mm. bore tubing extends from this stopcock to the outside of the warming jacket for the introduction of "test" perfusion fluids with little delay due to dead space because of the tubing size. The tubing below the stopcock is also of 2-mm. bore to reduce the dead space involved in switching back to the "normal" perfusion fluid.

In operation the various "test" solutions are prewarmed and oxygenated in a separate constant temperature bath. The perfusion fluid to be tested is suspended at the proper height and connected by pure gum rubber tubing to the side inlet on the warming jacket leading to the stopcock. The dead space (0.2 ml.) may be filled from a thin polyethylene tube attached to a syringe. By a 180° turn of the stopcock the "test" solution is supplied to the heart after a delay due to 0.45 ml. of dead space occupied by "normal" perfusate below the stopcock. Upon completion of the test or appearance of toxic effects, the "normal" perfusate may be rapidly restored to the heart by a second 180° turn of the stopcock.

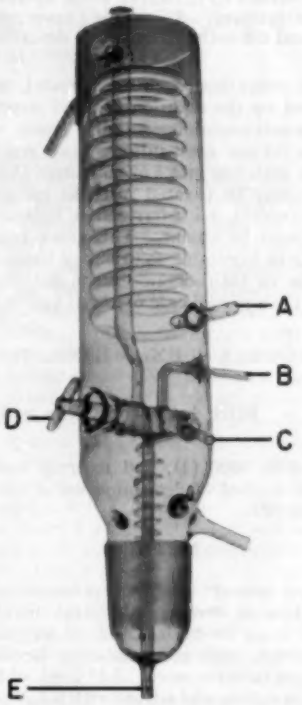

Fig. 1.—Photograph of Perfusion Chamber Modification. A—Inlet to warming spiral for "normal" perfusion solution, B—inlet for "Test" perfusion solution, C—inlet to capillary spiral for drug injection, D—three-way stopcock, E—heart cannula.

This apparatus has been used successfully with rabbit hearts without evidence of an insufficiency of the perfusing fluid due to the use of the small bore tubing. The modification has been found especially useful in the classic laboratory demonstration of the effect of inorganic cations and of alteration of substrates on the perfused isolated mammalian heart. A particular advantage is that these substances may be removed as well as added to the perfusion fluid in concentrations which may be correlated with *in vivo* blood concentrations.

* Received October 4, 1954, from the Department of Physiology and Pharmacology, and the Laboratory of the A. H. Robins Co., Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

[1] Supplied as Perfusion Chamber-Modification B; Cat. No. ME5512-Metro Industries, 29-28 Forty-first Ave., Long Island City 1, N. Y.

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A Preliminary Note on the Absorption of Sulfur and the Polythionates into Intact Skin*

By TORBEN E. NEESBY, ARNOLD KOFF†, and ANTHONY W. PIRCIO

AN INVESTIGATION has been undertaken to determine not only the penetrability of elemental sulfur and the polythionates through the skin barrier, but also to determine whether there is a relation between length of time of contact and percentage absorption of these sulfur preparations. In addition, experiments were conducted to compare the rate and amount of absorption of elemental sulfur and polythionates, and to determine the effect of various ointment vehicles on the rate and amount of absorption of the sulfur test compounds.

Preparation of Labeled Polythionates.—One millicurie of S^{35} in the form of elemental sulfur in benzene was added to a reaction flask containing 3.2 Gm. of carrier elemental sulfur, 100 ml. of water and 10 ml. of 10 *N* sodium hydroxide. The mixture was refluxed to obtain complete solution and then transferred to an acetylization flask and oxidized to thiosulfate by bubbling CO_2 free air through the solution for approximately twenty-four hours. Two milliliters of 10% ferric chloride was added as a catalyst for the reaction. After the iron sulfide coagulated and precipitated on standing, the solution was filtered into a 3-liter beaker and evaporated to dryness with the aid of an infrared lamp. Approximately 10 ml. of water was added to dissolve the residue and 6.4 Gm. of elemental iodine was added in order to oxidize the thiosulfate to tetrathionate. The sodium tetrathionate was crystallized from the solution by adding 200 ml. of alcohol with constant stirring. The yield of 5 Gm. of sodium tetrathionate was filtered off, washed with a minimum quantity of alcohol, and dissolved in 73 ml. of H_2O. Seventy-four milliliters of unlabeled Dermasulf,[1] which contained 2.71 Gm. pentathionate (as anion) and 2.26 Gm. tetrathionate (as anion) per 100 ml., was added to this solution. Since Dermasulf should have a *p*H of 1.0 ± 0.1, 1 ml. of concentrated HCl was added. Thus, the solution of S^{35} tagged polythionates had the following characteristics: *p*H 1.0 (±0.1) pentathionate (as anion) 1.36 Gm./100 cc.; tetrathionate (as anion) 3.57 Gm./100 cc. This radioactive polythionate solution had a specific activity of 25.2 c. p. m./1 mg.

Preparation of Labeled Elemental Sulfur.—Three and two-tenths Gm. of elemental sulfur, 10 ml. of 10 *N* NaOH, 100 ml. of water, and 1 millicurie of S^{35} in the form of elemental sulfur in benzene were placed in a reaction flask and refluxed until complete solution was obtained. The solution was cooled and dilute HCl was added to bring the *p*H to 7.0. The precipitated sulfur was collected on a filter paper and washed thoroughly with distilled water. The radioactive elemental sulfur preparation contained a specific activity of 920 c. p. m./mg.

Technique of Application of Various Sulfur Preparations.—Male adult rats of the Sprague-Dawley strain weighing from 200–265 Gm. were used throughout these studies. The interscapular area was carefully clipped of hair with an Oster clipper.

Two rats were used for the first phase of this study. After the animals were anesthetized, gauze pads about 2 cm. square in size were applied to adjacent areas of the clipped back of the rat. One pad was moistened with S^{35}-labeled 5% polythionate solution, and the other was moistened with non-radioactive 5% polythionate solution. At the end of 5, and 15 minutes the treated areas of the skin were carefully washed until free of activity as determined with a survey meter. Since the range of an S^{35} beta particle is only about 0.2 mm. in skin, the absorbed S^{35} could not activate the survey meter. The treated areas of the skin were removed from the back of the animal, and assayed for absorbed S^{35} by the method of Fingl, *et al.* (3). The counts were made with a scaler fitted with a 1.2 mg./cm.^{2} end-window Geiger tube.

In the second phase of the investigation, the absorption of 5% polythionate solution was compared with the absorption of 2% Dermasulf ointment, 3% aqueous precipitated elemental sulfur suspension, 2% sulfur in a U. S. P. hydrophilic ointment base (1), 2% sulfur in the U. S. P. sulfur ointment base (2), and 1.2% elemental sulfur in the Dermasulf Ointment base. The rats were prepared as described above. The ointments and the aqueous preparations were applied directly to the clipped area of the skin.

The animals were sacrificed after four hours, and the ointments and suspension were washed off the backs of the rats until no more radiation could be detected. The rat, which had been treated with 5% polythionate solution, was left unwashed. Approximately 50 x 50 mm. portions of the treated skin were removed, weighed, and the exact area determined.

After removing a skin sample from the unwashed rat, the remainder of the treated skin of the rat was washed, and a piece removed and numbered. The skin samples were assayed for S^{35} by the aforementioned procedure (3).

SUMMARY

A method has been described for the study of the absorption of elemental sulfur and the polythionates into the intact skin of rats.

* Received December 20, 1954, from the Research Laboratories of Carroll Dunham Smith Pharmacal Company, New Brunswick, N. J.

† Present address: Hoffmann-La Roche, Inc., Nutley, N. J.

[1] Trademark of Carroll Dunham Smith Pharmacal Company, an ethical, stabilized, 5% acid polythionate solution for topical application.

REFERENCES

(1) "U. S. Pharmacopeia," 14th rev. Mack Publishing Co., Easton, Pa., 1950, p. 391.

(2) "U. S. Pharmacopeia," 14th rev., Mack Publishing Co., Easton, Pa., 1950, p. 595.

(3) Fingl, E. G., Christian, J. E., and Edwards, L. D., THIS JOURNAL, **39**, 694(1950).

A Note on Serum Levels and Excretion of Salicylamide in Man*

By JOHN HIDALGO and VICTOR P. SEEBERG, with the Technical Assistance of JOANNE GUL DENZOPF

CRAMPTON AND VOSS (1) recently reported the results of an investigation of the absorption excretion and blood levels of salicylamide in rabbits, and concluded that salicylamide was absorbed and excreted more rapidly than sodium salicylate. They found salicylamide blood levels to reach a peak one hour after oral administration and to fall rapidly thereafter, the blood being practically free of drug in three or four hours. Urinary excretion was also rapid; 80 per cent of a dose was recovered from the urine four hours after administration. They employed an assay method which measured both free and combined salicylamide.

Seeberg, *et al.* (2), obtained similar results in rabbits and rats. Using somewhat larger doses, peak serum levels were obtained one-half hour after oral administration, which then fell to low levels by the fourth and fifth hours. Levels in muscle and brain were found roughly to parallel serum levels. With the same dose of aspirin, peak serum levels were not reached until two hours after drug administration with no appreciable fall from maximum levels at five hours. Peak levels in muscle and brain were not attained until three to five hours after administration of aspirin. An assay method was employed which measured only the uncombined drug. Therefore, peak serum levels were considerably lower with salicylamide than those reported by Crampton and Voss.

The rapid absorption, early peak serum levels, and rapid excretion of salicylamide in animals observed by the two above groups of investigators do not agree with the high and prolonged blood levels reported by Euler and Remy (3) in human subjects, three and four hours after oral administration of 3 Gm. of salicylamide. Crampton and Voss, therefore, questioned whether conclusions regarding the rate of absorption and excretion in rabbits could be applied to man.

To clarify this point, we administered 2 Gm. of salicylamide orally to five healthy adult volunteers weighing 120 to 160 pounds, and studied serum levels as well as urinary excretion. Three subjects were males and two were females. Each subject ate a light breakfast about 7:30 a. m. The drug was administered at 10 a. m. and lunch was eaten between 12 and 1 p. m. Thus, drug administration was made on a stomach containing a certain amount of food. A blood sample was obtained from each subject just before drug administration to serve as a blank in the assay of subsequent samples. Since it was felt undesirable to make more than four consecutive venipunctures on each subject, blood samples at three intervals from one-half to four hours after administration were obtained. Urinary excretion was determined at four and six hours after administration. The assay procedure used was the same as that used by Crampton and Voss, except that 3 cc. of serum was used instead of 1 cc. in order to increase the sensitivity of the method. The results are summarized in Table I.

* Received November 15, 1954, from the Research Division, Cutter Laboratories, Berkeley, Calif.

SUMMARY

Although the series is small, certain points which we were particularly interested in elucidating seem rather clear. Blood serum levels rose rapidly to a maximum mean value of 4.85 mg. % at one hour after drug administration and fell to approximately one-fourth the peak value or a mean value of 1.27 mg. % by four hours. This is in marked contrast to the data of Smith, *et al.* (4), on aspirin in man. Blood levels following the same dose of aspirin did not reach their peak until two hours after administration and had not fallen appreciably from peak values at four and six hours.

The mean urinary excretion of salicylamide during the first four hours following administration was 43.8% in the five subjects studied. This is relatively rapid excretion and is again in marked contrast to excretion data on aspirin. Following the administration of 1.95 Gm. of aspirin, Lester, *et al.* (5), found a urinary excretion of only about 11% of the administered dose during the first four hours.

These data indicate that rapid absorption, early peak serum levels and rapid excretion are characteristic of salicylamide in both animals and man.

We have no satisfactory explanation for the high serum levels in human subjects three and four hours after the administration of salicylamide reported by Euler and Remy. They administered a 3-Gm. dose, whereas we administered 2 Gm., but it does not seem possible that a 50% increase in dose could be responsible for such a marked and prolonged elevation of serum levels. Conclusions regarding the rate of distribution of salicylamide into tissues other than blood in man must be drawn by analogy from the animal studies, since it is not possible to obtain such data directly.

REFERENCES

(1) Crampton, J. M., and Voss, E., THIS JOURNAL, **48**, 470(1954).

(2) Seeberg, V. P., Hansen, D., and Whitney, B., *J. Pharmacol. Exptl. Therap.*, **101**, 275(1951).

(3) Euler, E., and Remy, R., *Med. Klin., (Munich)*, **45**, 1178(1950).

(4) Smith, P. K., Gleason, H. L., Stoll, C. B., and Orgorzalek, S., *J. Pharmacol. Exptl. Therap.*, **87**, 237(1946).

(5) Lester, D., Lolli, G., and Greenberg, L. A., *ibid.*, **87**, 329(1946).

TABLE I.—SERUM LEVELS AND URINARY EXCRETION OF SALICYLAMIDE IN MAN AFTER 2 GM. ORALLY

Serum Levels, mg. %				Urinary Excretion		
Time, hr.	No. of Analyses	Mean	Range	Time, Hr.	Total Dose Excreted, % Mean	Range
½	2	1.55	1.4–1.7			
1	4	4.85	3.5–6.3			
2	4	3.22	3.1–3.3	0–4	43.8	36–50
4	4	1.27	0.5–2.1	4–6	4.1	3–12

Book Notices

Small-Scale Inorganic Qualitative Analysis. By J. T. Stock and J. Heath. Chemical Publishing Co., Inc., New York, 1954. 96 pp. 12.5 x 18.7 cm. Price $2.50.

The classical "six-group" method of qualitative analysis, modified for application to centigram or semi-micro scale procedure, is described in this small book. The text is concisely written and includes illustrated descriptions of the apparatus used. Most liquid transfers are made with "teat pipettes" (long-tipped droppers), separations utilize the centrifuge rather than filtration, and simple gas pressure systems replace bubbling hydrogen sulfide gas. The book can serve as a laboratory guide for the instructur in qualitative analysis. Appendixes give approximate quantities of chemicals required to prepare reagents and solutions for analytical practice.

Methoden der Organischen Chemie (Houben-Weyl). 4th ed. Vol. 4, Part 2. Allgemeine chemische Methoden. B. Helferich, H. Henecka, G. Hesse, H. Koch, J. Krebs, H. Kröper, F. Müller, W. Reisner, G. Schiller, H. Simon, K. Stetter, W. Theilacker, F. Weygand, K. Wimmer, K. Ziegler, Editors. Georg Thieme Verlag, Stuttgart, 1955. XXVIII + 1004 pp. 19 x 26 cm. Price $36.20.

This volume of *Houben-Weyl* is the fourth in the series constituting the complete revision of this important reference manual. The volumes constituting the new revision are not appearing in numerical sequence. For a short résumé of the three earlier volumes, see This Journal, **43**, 447(1954). The latest volume covers the subject of catalysis in a comprehensive manner. About one-half of the book is devoted to a treatment of catalytic processes, including a survey of the entire field of homogeneous and heterogeneous reactions. Methods of preparation of catalysts, descriptions of the different types of apparatus, and general procedures used in heterogeneous catalytic organic reactions are stressed.

The section on catalysis is introduced by two short chapters in which homogeneous catalysis is discussed and interpreted in a theoretical manner.

The section on catalysis is followed by a discussion of pyrochemical processes, electrochemical procedures, the preparation of optically-active compounds, and a comprehensive treatment of the synthesis of isotopes. Other procedures are described for the preparation and conversion of large ring systems. The methods of preparing such ring compounds are theoretically interesting from a preparative standpoint and they are discussed with this in mind. Volume IV (2) is concluded by two chapters discussing general methods of biochemistry and microbiological chemical working techniques. Here organic rather than biochemistry is particularly emphasized. Documentation includes references to the scientific and to the most important patent literature up to the middle of 1954. The book is provided with an adequate index which is not so detailed as to present difficulties in its use.

50 Jahre Arzneimittelforschung. By C. L. Lautenschlager. Georg Thieme Verlag, Stuttgart, 1955. 486 pp. 18 x 25 cm. Price $8.60.

Few writers are in the fortunate position of the author of this interesting and stimulating book. He has been active as a pharmacist, a physician and a chemist in retail pharmacy, industry, and teaching positions for over fifty years—the period covered by the historical presentation which represents the main theme of his work. The book is, however, not limited to a description of past developments, but presents a lucid survey of the present state of research and practice. Chemotherapy, hormones, organotherapeutic preparations, narcotics and other pharmaceuticals are taken up systematically and with equal adeptness.

Professor Lautenschlager deplores the fact that the pharmacist is not given proper educational opportunities to familiarize himself sufficiently with the allied fields of medicine and biology to be able to keep abreast of the rapidly changing ideas—and practices derived therefrom—in today's therapy. His book leaves no doubt about his sovereign mastery of all these fields.

The reference value of the work suffers from lack of an index which is only partly compensated by a detailed table of contents.

Methods of Biochemical Analysis. Vol 2. Edited by David Glick. Interscience Publishers, Inc., New York, 1955. vi + 470 pp. 16 x 23.5 cm. Price $9.50.

This is the second of an annual series, the first of which was published in 1954, in which authoritative methods, procedures and techniques for the determination and assaying of biologically important substances and systems are presented. The first of the series was reviewed in This Journal, **43**, 445(1954).

Volume II of *Methods of Biochemical Analysis* is divided into thirteen chapters. The arrangement of each chapter, in general, follows the design employed in Volume I.

The first chapter of Volume II, "Analysis of Steroids by Infrared Spectrometry" is divided into seven sections, including an introduction, a discussion of the principles of infrared spectrometry, steroidal nomenclature, the preparation of samples, the correlation of steroid structure and infrared absorption, analytical procedures applicable to isolated or synthesized steroids by infrared spectrometry, and a summary followed by a list of more than seventy references extending through the greater part of 1954. Other chapters follow essentially the same design wherever applicable, with a discussion of the background of previous work, a critical evaluation of the various approaches, and a presentation of methods of analysis that have been employed and found to be satisfactory. These other chapters present methods of analysis for such biologically important compounds as epinephrine and norepinephrine in body fluids and tissues; lipide analysis; determination of lipoxidase activ-

ity; the assay of compounds with folic acid activity; methods for the determination of tocopherols (vitamin E); methods for the determination of coenzyme A; the assay of proteolytic enzymes; the determination of glutathione, serum glycoproteins, sugars in polysaccharides; recent developments in techniques for terminal and sequence studies in peptides and proteins; and in the final chapter, the spectrophotometric assay of cytochrome c oxidase.

In each of the several chapters, experimental details are presented so as to furnish the research worker with sufficient information to carry out any analysis discussed. The book is documented throughout with references to the original literature and is provided with author and subject indices. Many of the methods described in Volume I are employed in the standardization of pharmaceutical products, and thus the book should be of special interest to analysts and research workers in the pharmaceutical industry.

Organic Syntheses. Collective Vol. 3. A revised edition of annual volumes 20–29. E. C. HORNING, Editor-in-Chief. John Wiley & Sons, Inc., New York, (Chapman & Hall, Ltd., London) 1955. x + 890 pp. 16 x 23.5 cm. Price $15.

It is now more than thirty years since the first annual volume of the Organic Syntheses series made its appearance. During these years, it has become firmly established as an authoritative reference for the preparation of many organic chemical compounds. By 1932, the series had become so well established that it seemed desirable to consolidate the first nine volumes into a single collective book. The second collective volume, covering volumes 10–19 was published in 1934.

Continuing the practice started in 1932, volumes 20–29 have now been combined into collective Volume III. In this volume, the editors have made necessary revisions from the original material, eliminating errors found in the original printing in single volume form, and correcting where necessary calculations and references. Some modifications and improvements in procedures have been incorporated, and in instances where manufacturing chemical progress has made the products described in the annual volumes available cheaply, this fact has been indicated, but full details for the preparation of such compounds have been continued.

Literature references have been reviewed and brought up to date through Volume 46, 1952, of *Chemical Abstracts*. There has also been added to each preparation as a subtitle, the indexing name used by *Chemical Abstracts* when that name differs from the one commonly employed.

Collective Volume III of Organic Syntheses is provided with a type of reaction index, a type of compound index, a formula index, an index to preparation or purification of solvents and reagents, an illustration index, and a general index, which enhance considerably the reference value of the work

The Rauwolfia Story. From primitive medicine to alkaloidal therapy. Edited and published by CIBA PHARMACEUTICAL PRODUCTS, INC., Summit, New Jersey, 1954. 63 pp. 11 x 18.5 cm.

This little book, prepared under the direction of Ciba Pharmaceutical Products, Inc., describes in chronological order the dramatic story of the development of Rauwolfia therapy. The book is divided into three parts, in the first of which the early Indian and European history of *Rauwolfia serpentina* is presented. In Part II, entitled "Rauwolfia Comes West," the history of the drug during the past twenty years is reviewed, and in the final chapter Rauwolfia therapy of today is covered. The story of Rauwolfia is written in an entertaining and informative manner, and should be of interest to pharmacists and others who wish to acquire a background knowledge of Rauwolfia and its principal derivatives.

BOOKS RECEIVED

Antibiotics Annual, 1954–1955. Proceedings of the Second Annual Symposium on Antibiotics. HENRY WELCH and FELIX MARTI-IBANEZ, Editors. Medical Encyclopedia, Inc., New York, 1955. ix + 1154 pp. 18 x 26 cm. Price $10.

Collectanea Pharmaceutica Suecica. Vol. 9. Kungl. Farmaceutiska Institutets Bibliotek, Stockholm, 1954. (11 articles.) 15 x 22.7 cm.

1955 Medical Progress. A Review of Medical Advances During 1954. MORRIS FISHBEIN, Editor. The Blakiston Division, McGraw-Hill Book Company, Inc., New York, 1955. 12 + 346 pp. 16 x 23.7 cm. Price $5.

The Pharmacological Basis of Therapeutics. 2nd ed. By LOUIS S. GOODMAN and ALFRED GILMAN. The Macmillan Company, New York, 1955. 13 + 1832 pp. 18.5 x 24 cm. Price $17.50.

The Role of Humoral Agents in Nervous Activity. By BRUNO MINZ. Charles C Thomas, Springfield, Illinois, 1955. viii + 230 pp. 15 x 22.5 cm. Price $7.75.

Modern Gas Analysis. By PAUL W. MULLEN. Interscience Publishers, Inc., New York, 1955. ix + 354 pp. 12.5 x 18.5 cm. Price $5.50.

New Practical Formulary. By MITCHELL FREEMAN. Chemical Publishing Company, Inc., New York, 1955. 376 pp. 14 x 21.5 cm. Price $7.95.

Introduction to Theoretical Organic Chemistry. By P. H. HERMANS (Utrecht). Edited and revised by R. E. REEVES. Elsevier Press, Inc., Houston, 1955. xii + 507 pp. 16.5 x 23.5 cm. Price $9.75.

Management of Addictions. Edited by EDWARD PODOLSKY. Philosophical Library, Inc., New York, 1955. xvii + 413 pp. 15 x 22.3 cm. Price $7.50.

Practical Endocrinology. By LEWIS M. HURXTHAL. McGraw-Hill Book Co., New York, 1955. xii + 318 pp. 14 x 21 cm.

Technique of Organic Chemistry. Vol. 7. Edited by ARNOLD WEISSBERGER. Interscience Publishers, Inc., New York, 1955. vii + 552 pp. 16 x 24.75 cm. Price $8.50.

World Health Organization Recommended International Non-Proprietary Names

The following names in accordance with paragraphs 3A and 7 of the Procedure of the Selection of Recommended International Non-Proprietary Names for Drugs moving in International Commerce, will be published in a forthcoming issue of the *Chronicle of the World Health Organization.* Comments on, or formal objections to, the above names may be filed within a period of six months from July 1, 1955, and should be forwarded to the Director-General, World Health Organization, Palais des Nations, Geneva, Switzerland.

A formal objection to a proposed name must include the identity of the person objecting, a statement of his interest in the name, and a statement setting forth the reasons for his objection to the name proposed.

Recommended International Non-Proprietary Names (Latin, English)	Chemical Names or Description
acebrocholum acebrochol	acetodibromodihydrocholesterol
acetaminosalolum acetaminosalol	acetyl-4-aminophenyl salicylate
acidum aminoaceticum amino-acetic acid	amino-acetic acid, glycocoll
acidum dehydrocholicum dehydrocholic acid	
acidum gentisicum gentisic acid	5-hydroxysalicylic acid
acidum glutamicum glutamic acid	glutamic acid
acidum iopanoicum iopanoic acid	3-(3-amino-2,4,6-triiodophenyl)-2-ethylpropanoic acid
acriflavinii chloridum acriflavinium chloride	mixture of the hydrochlorides of 3,6-diamino-10-methylacridinium chloride and 3,6-diamino-acridine
aethacridinum ethacridine	2-ethoxy-6,9-diaminoacridine
aethoxazorutosidum ethoxazorutoside	monomorpholylethylrutoside
alcoholum benzylicum benzyl alcohol	phenylcarbinol
aldesulfonum natricum aldesulfone sodium	disodium salt of 4,4′-diaminodiphenylsulfone formaldehydesulfoxylic acid
allobarbitalum allobarbital	5,5-diallylbarbituric acid
allocupreidum natricum allocupreide sodium	sodium cuproallylthioureidobenzoate
allomethadionum allomethadione	3-allyl-5-methyloxazolidine-2,4-dione
aminoaethylis nitras aminoethyl nitrate	nitrate ester of aminoethanol
aminothiazolum aminothiazole	
amodiaquini hydrochloridum amodiaquine hydrochloride	7-chloro-4-(3′-diethylaminomethyl-4′-hydroxyanilino) quinoline dihydrochloride dihydrate
antazolini hydrochloridum antazoline hydrochloride	2-N-benzylanilinomethylimidazoline hydrochloride
aprobarbitalum aprobarbital	allylisopropylbarbituric acid
aurothioglycanidum aurothioglycanide	auromercaptoacetanilide
azamethonii bromidum azamethonium bromide	3-methyl-3-azapentane-1,5-bis(ethyldimethyl-ammonium) bromide
bacitracinum bacitracin	
benethamini penicillinum benethamine penicillin	benzylpenicillin salt of N-benzyl-β-phenylethylamine
benzalkonii chloridum benzalkonium chloride	mixture of alkylbenzyldimethylammonium chlorides
benzethonii chloridum benzethonium chloride	benzyldimethyl-*p*-(1,1,3,3-tetramethylbutyl) phenoxyethoxyethylammonium chloride
benzododecinium benzododecinium	dimethylbenzyldodecylammonium
benzoestrolum benzoestrol	3-ethyl-2,4-bis(*p*-hydroxyphenyl) hexane
benzpyrinii bromidum benzpyrinium bromide	1-benzyl-3-(dimethylcarbamyloxy) pyridinium bromide

(W. H. O. NON-PROPRIETARY NAMES *cont'd*)

bibrocatholum bibrocathol	bismuth derivative of tetrabromopyrocatechol
camphotamidum camphotamide	camphosulfonyl-*N*-methylpyridine-β-diethylcarbonamide
caramiphenii chloridum caramiphenium chloride	diethylaminoethyl-1-phenylcyclopentane-1-carboxylate hydrochloride
carbromalum carbromal	α-bromo-α-ethylbutyrylurea
cetobemidonum ketobemidone	4-*m*-hydroxyphenyl-1-methyl-4-propionoylpiperidine
cetrimonium cetrimonium	cetyltrimethylammonium
cetylpyridinii chloridum cetylpyridinium chloride	
chloramphenicolum chloramphenicol	D(−)-threo-1-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol
chlorazodinum chlorazodin	α,α-azo-bis(chloroformamidine)
chlornaphazinum chlornaphazine	2,2′-dichlorodiethyl-β-naphthylamine
chloropyrilenii citras chloropyrilenium citrate	N,N-dimethyl-N′-(2-pyridyl)-N′-(5-chloro-2-thenyl) ethylenediamine citrate
chlorquinaldolum chlorquinaldol	5,7-dichloro-8-hydroxy-2-methylquinoline
cinchocainii chloridum cinchocainium chloride	hydrochloride of the β-diethylaminoethylamide of 2-butyloxycinchoninic acid
cinchophenum cinchophen	2-phenylquinoline-4-carboxylic acid
cocarboxylasum cocarboxylase	pyrophosphoric ester of thiamine
corticotrophinum corticotrophin	adrenocorticotrophic hormone
cyanocobalaminum cyanocobalamin	vitamin B_{12}
cyclobarbitalum cyclobarbital	5-(1-cyclohexenyl)-5-ethylbarbituric acid
cyclopentaminum cyclopentamine	N,-α-dimethylcyclopentylethylamine
cyclopropanum cyclopropane	
decamethonium decamethonium	decamethylene-1,10-bistrimethylammonium
diaphenylsulfonum diaphenylsulfone	4,4′-diaminodiphenylsulfone
dichlorophenarsini hydrochloridum dichlorophenarsine hydrochloride	3-amino-4-hydroxyphenyl dichlorarsine hydrochloride
diethylcarbamazinum diethylcarbamazine	1-diethylcarbamoyl-4-methylpiperazine
digitoxosidum digitoxoside	one of the heterosides of the leaf of *Digitalis purpurea* L.
dihydrostreptomycinum dihydrostreptomycin	
dihydrotachysterolum dihydrotachysterol	
diiodohydroxyquinolinum diiodohydroxyquinoline	8-hydroxy-5,7-diiodoquinoline
dimenhydrinatum dimenhydrinate	2-(diphenylmethoxy)-N,N-dimethylethylammonium 8-chlorotheophyllinate
dimercaprolum dimercaprol	2,3-dimercaptopropanol
dimethiodalum natricum dimethiodal sodium	sodium diiodomethanesulfonate
dimethyltubocurarinii chloridum dimethyltubocurarinium chloride	
dimoxylinii phosphas dimoxylinium phosphate	6,7-dimethoxy-1-(4′-ethoxy-3′,ethoxy-3′-methoxybenzyl) 3-methylisoquinolinium phosphate
diperodoni hydrochloridum diperodon hydrochloride	3-(1-piperidyl)1,2-propanediol dicarbanilate hydrochloride
diphenanum diphenan	4-benzylphenyl carbamate
diphenhydramini hydrochloridum diphenhydramine hydrochloride	β-benshydryl 2-dimethylaminoethyl ether hydrochloride
diprophyllinum diprophylline	dihydroxypropyltheophylline
disulfiramum disulfiram	tetraethylthiuram disulfide

W. H. O. NON-PROPRIETARY NAMES *cont'd.*

dixanthogenum dixanthogen	diethyldixanthogen
doxylaminii succinas doxylaminium succinate	2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl] pyridine succinate
ethinyloestradiolum ethinyloestradiol	17-ethinyl-3,17-dihydroxy-Δ-1,3,5-oestratriene
eucatropinum eucatropine	4-hydroxy-1,2,2,6-tetramethylpiperidine phenyl-glycollate
fumagillinum fumagillin	an antibiotic substance produced by certain strains of *Aspergillus fumigatus*
furtrethonii iodidum furtrethonium iodide	furfuryltrimethylammonium iodide
glucosulfamidum glucosulfamide	glucose sodium bisulfite compound of sulfanilamidomethanol
glucosulfonum glucosulfone	*p,p'*-diaminodiphenylsulfone-N,N'-di-(glucose sodium sulfonate)
gonadotrophinum chorionicum chorionic gonadotrophin	contains the gonad-stimulating substance obtained from the urine of pregnant women
gonadotrophinum sericum serum gonadotrophin	contains the follicle-stimulating substance obtained from the serum of pregnant mares
gramicidinum gramicidin	
heptaminolum heptaminol	2-amino-6-methylheptan-6-ol
hexachlorophenum hexachlorophene	di-(2-hydroxy-3,5,6-trichlorophenyl) methane
hexamethonium hexamethonium	hexamethylene-1,6-bistrimethylammonium
hexobarbitalum hexobarbital	5-(1-cyclohexenyl)-1,5-dimethylbarbituric acid
hexoestrolum hexoestrol	3,4-di-(*p*-hydroxyphenyl)*n*-hexane
homatropini methylbromidum homatropine methylbromide	
hyaluronidasum hyaluronidase	enzymes of various origins which depolymerize hyaluronic acid
hydralazinum hydralazine	1-hydrazinophthalazine
hydrocodoni bitartras hydrocodone bitartrate	dihydrocodeinone acid tartrate
hydromorphoni hydrochloridum hydromorphone hydrochloride	dihydromorphinone hydrochloride
hydroxyprocainum hydroxyprocaine	diethylaminoethanol 4-aminosalicylate
hydroxypyridini tartras hydroxypyridine tartrate	tartrate ester of 3-hydroxypyridine
hydroxytetracainum hydroxytetracaine	2-dimethylaminoethanol 4-*n*-butylamino-salicylate
ibrotamidum ibrotamide	ethylisopropyl-α-bromacetamide
iodetrylum iodetryl	ethyl diiodostearate
iodophthaleinum natricum iodophthalein sodium	disodium salt of tetraiodophenolphthalein
iodothiouracilum iodothiouracil	4-hydroxy-5-iodo-2-mercaptopyrimidine
iproniazidum iproniazid	1-isonicotinyl-2-isopropylhydrazide
isomethadonum isomethadone	6-dimethylamino-4,4-diphenyl-5-methyl-3-hexanone
isoprenalinum isoprenaline	1-(3',4'-dihydroxyphenyl)2-*iso*propylaminoethanol
khellinum khellin	5,8-dimethoxy-3-methyl-6,7-furanochromone, extracted from the fruits of *Ammi visnaga* (L.) Lam.
levomethorphanum levomethorphan	(−) 3-methoxy-*N*-methylmorphinan
maleylsulfathiazolum maleylsulfathiazole	maleyl-*p*-aminophenylsulfonamidothiazole
medrylaminum medrylamine	β-dimethylaminoethyl-*p*-methoxybenzhydryl ether
mephenesinum mephenesin	1,2-dihydroxy-3-(2'-methylphenoxy) propane
mepyraminum mepyramine	N-*p*-methoxybenzyl-N',N'-dimethyl-N-2-pyridylethylenediamine
meralluridum meralluride	mixture of methoxyoxymercuripropylsuccinylurea and theophylline

(W. H. O. NON-PROPRIETARY NAMES *cont'd*)

merbrominum merbromin	disodium salt of 2,7-dibromo-4-hydroxy-mercurifluoresceine
mercaptaminum mercaptamine	β-mercaptoethylamine
mercaptomerinum mercaptomerin	disodium salt of N(3-carboxymethylmercaptomercuri-2-methoxy) propylcamphoramic acid
mercuderamidum mercuderamide	hydroxymercuripropanolamide of *o*-carboxyphenoxyacetic acid
mercurobutolum mercurobutol	4-*tert*-butyl-2-chloromercuriphenol
mercurophyllinum mercurophylline	mixture of the sodium salt of the β-methoxy-γ-hydroxymercuripropylamide of trimethylcyclopentanedicarboxylic acid and theophylline
metaraminoli bitartras metaraminol bitartrate	(−) 1-*m*-hydroxyphenyl-2-amino-1-propanol hydrogen (+) tartrate
methacholinii chloridum methacholinium chloride	acetyl-β-methylcholinium chloride
methandriolum methandriol	17α-methyl-3β,17β-dihydroxyandrostene-5
methaphenilenum methaphenilene	N,N-dimethyl-N-phenyl-N-(2-thienylmethyl) ethylenediamine
methapyrilenum methapyrilene	N,N-dimethyl-N′-(2-pyridyl)-N′-(2-thienyl) ethylenediamine
metharbitalum metharbital	5,5-diethyl-1-methyl barbituric acid
methenaminii tetraiodidum] methenaminium tetraiodide	hexamethylenetetraminium tetraiodide
methenaminum methenamine	hexamethylenetetramine
methestroli dipropionas methestrol dipropionate	4,4′-(1,2-diethylethylene)di-*o*-cresol dipropionate
methiodalum natricum methiodal sodium	sodium iodomethanesulfonate
methylbenzethonii chloridum methylbenzethonium chloride	benzyldimethyl-2-[2-(*p*-1,1,3,3-tetramethyl-butyl-cresoxy) ethoxy] ethylammonium chloride
methylergometrinii tartras methylergometrinium tartrate	D-lysergic acid 2-butanolamide tartrate
methylphenobarbitalum methylphenobarbital	N-methyl-5-ethyl-5-phenylbarbituric acid
methylrosanilinii chloridum methylrosanilinium chloride	crystal violet
methylthioninii chloridum methylthioninium chloride	tetramethylthioninium chloride
methylthiouracilum methylthiouracil	4-methyl-2-thiouracil
monoaethanolaminii oleas monoethanolaminium oleate	
nalorphinum nalorphine	N-allylnormorphine
naphazolinum naphazoline	2-(1-naphthylmethyl) imidazoline
natrii ascorbas sodium ascorbate	
natrii aurothiomalas sodium aurothiomalate	mainly the sodium salt of aurothiomalic acid
natrii cyclamas sodium cyclamate	sodium cyclohexylsulfamate
natrii dehydrocholas sodium dehydrocholate	
natrii gentisas sodium gentisate	sodium 5-hydroxysalicylate
natrii morrhuas sodium morrhuate	sodium salts of the fatty acids of cod-liver oil
natrii stibogluconas sodium stibogluconate	sodium antimonylgluconate
natrii tetradecylis sulfas sodium tetradecyl sulfate	sodium 7-ethyl-2-methylundecyl-4-sulfate
nicopholinum nicopholine	morpholine nicotinic acid amide
nitrosulfathiazolum nitrosulfathiazole	2-(*p*-nitrophenylsulfonamido) thiazole
octamylaminum octamylamine	isoamylaminomethylheptane
oxapropanii iodidum oxapropanium iodide	1-dimethylaminomethylene-2,3-dioxypropane iodomethylate
oxophenarsini hydrochloridum oxophenarsine hydrochloride	3-amino-4-hydroxy-phenyl arsenoxide hydrochloride

(W. H. O. NON-PROPRIETARY NAMES *cont'd*)

oxycodoni hydrochloridum oxycodone hydrochloride	dihydrohydroxycodeinone hydrochloride
oxydipentonii chloridum oxydipentonium chloride	5,5′-bis(trimethylammoninium) dipentyl ether dichloride
oxyphenonii bromidum oxyphenonium bromide	diethyl-2(-hydroxyethyl)methylammonium bromide phenyl-cyclohexyl-glycolate
oxytetracyclinum oxytetracycline	an antibiotic substance produced by the growth of *Streptomyces rimosus*, or the same substance produced by any other means
paramethadionum paramethadione	3,5-dimethyl-5-ethyloxazolidine-2,4-dione
parethoxycainum parethoxycaine	diethylaminoethanol 4-ethoxybenzoate
paroxypropionum paroxypropione	4-hydroxypropiophenone
pentaerithrityli tetranitras pentaerithrityl tetranitrate	
pentamethonium penthamethonium	pentamethylene-1,5-bistrimethylammonium
pentamidinum pentamidine	*p-p′*-diamidino-diphenoxypentane
pentobarbitalum pentobarbital	5-ethyl-5-(1-methylbutyl) barbituric acid
phenacemidum phenacemide	phenylacetylurea
phenadoxonum phenadoxone	6-morpholino-4,4-diphenylheptan-3-one
phenicarbazidum phenicarbazide	phenylsemicarbazide
phenindaminum phenindamine	2-methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene
pheniodolum natricum pheniodol sodium	sodium-α-phenyl-α-(4-hydroxy-3,5-diiodophenyl) propionate
phenododecinium phenododecinium	dodecyldimethyl-2-phenoxyethylammonium
phenothiazinum phenothiazine	
phentolaminum phentolamine	2-(*m*-hydroxy-*N*-*p*-tolylanilinomethyl)-2-imidazoline
phenylbutazonum phenylbutazone	3,5-dioxo-1,2-diphenyl-4-*n*-butylpyrazolidine
pholcodinum pholcodine	morpholinylethylmorphine
pholedrinii sulfas pholedrinium sulfate	β-(*p*-hydroxyphenyl) isopropylmethylammonium sulfate
phthalylsulfathiazolum phthalylsulfathiazole	(*o*-carboxybenzoyl)-*p*-aminophenylsulfonamido-thiazole
piperocainii chloridum piperocainium chloride	3-benzoxy-1-(2-methylpiperidino) propane hydrochloride
piperoxani hydrochloridum piperoxan hydrochloride	2-(1-piperidylmethyl)1,4-benzodioxan hydrochloride
piridocainum piridocaine	β-(2-piperidyl) ethyl *o*-aminobenzoate
pregnenolonum pregnenolone	3-hydroxy-20-keto-pregnene-5
primaquinum primaquine	8-(4-amino-1-methylbutylamino)-6 methoxyquinoline
probarbitalum natricum probarbital sodium	sodium derivative of 5-ethyl-5-isopropylbarbituric acid
procainamidum procainamide	4-amino-(2-diethylaminoethyl) benzamide
procyclidinum procyclidine	1-phenyl-1-*cyclo*hexyl-3-pyrrolidinopropan-1-ol hydrochloride
promethazini hydrochloridum promethazine hydrochloride	(dimethylamino-2′-methyl-2′-ethyl)-*N*-dibenzoparathiazine hydrochloride
propamidinum propamidine	α,ω-(4,4′-diamidinodiphenoxy) propane
propanthelini bromidum propantheline bromide	β-diisopropylaminoethyl xanthene-9-carboxylate methylbromide
propyliodonum propyliodone	propyl-3,5-diiodo-4-pyridone acetate
propylthiouracilum propylthiouracil	2-mercapto-4-hydroxy-6-*n*-propylpyrimidine
propyphenazonum propyphenazone	1-phenyl-2,3-dimethyl-4-isopropyl-5-pyrazolone
pyridoxinii chloridum pyridoxinium chloride	4,5-di(hydroxymethyl)-3-hydroxy-2-methyl-pyridinium chloride

(W. H. O. NON-PROPRIETARY NAMES *cont'd*)

pyrimethaminum pyrimethamine	2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine
pyroxylinum pyroxylin	soluble guncotton
racemethorphanum racemethorphan	(±) 3-methoxy-*N*-methylmorphinan
racemorphanum racemorphan	(±) 3-hydroxy-*N*-methylmorphinan
rutosidum rutoside	3-rhamnoglucoside of 5,7,3′,4′-tetrahydroxy-flavonol
salacetamidum salacetamide	*N*-acetylsalicylamide
salazosulfamidum salazosulfamide	*p*-sulfonamidophenylazosalicylic acid
salazosulfathiazolum salazosulfathiazole	*p*-aminophenylsulfonamidothiazoleazosalicylic acid
saicylamidum salicylamide	2-hydroxybenzamide
solutio natrii chloridi composita compound solution of sodium chloride	synonym: Ringer's solution
solutio natrii lactatis composita compound solution of sodium lactate	synonym: Ringer's lactate solution
stearylsulfamidum stearylsulfamide	stearylsulfanilamide
stibamini glucosidum stibamine glucoside	N-glucoside of sodium 4-aminophenylstibonate
streptomycinum streptomycin	
subathizonum subathizone	4-ethylsulfonylbenzaldehyde thiosemicarbazone
sulfacetamidum sulfacetamide	*p*-aminophenylsulfonacetamide
sulfadimidinum sulfadimidine	2-sulfanilamido-4,6-dimethylpyrimidine
sulfisomidinum sulfisomidine	2,4-dimethyl-6-sulfanilamidopyrimidine
sulfogaiacolum sulfogaiacol	potassium guaiacolsulfonate
suraminum natricum suramin sodium	symmetrical area of the sodium salt of *m*-benzoyl-*m*-amino-*p*-methyl-benzoyl-1-aminonaphthalene 4,6,8-trisulfonic acid
suxethonii chloridum suxethonium chloride	bis-(2-dimethylaminoethyl) succinate bisethochloride
thiacetarsamidum natricum thiacetarsamide sodium	disodium salt of *p*-[bis-(carboxymethylmercapto) arsino]benzamide
thiazosulfonum thiazosulfone	2,4′-diaminothiazolyphenylsulfone
thioacetazonum thioacetazone	4-acetamidobenzaldehyde thiosemicarbazone
thiodiglycolum thiodiglycol	2,2′-dihydroxyethyl sulfide
thonzylaminii chloridum thonzylaminium chloride	N,N-dimethyl-N′-(*p*-methoxybenzyl)-N′-(2-pyrimidyl) ethylenediamine hydrochloride
tocamphylum tocamphyl	diethanolamine salt of tolylmethyl carbinol mono-*d*-camphoric acid ester
tolazolinum tolazoline	2-benzylimidazoline
trichloroethylenum trichlorethylene	
trihexyphenidyli hydrochloridum trihexyphenidyl hydrochloride	1-cyclohexyl-1-phenyl-3-piperidino-1-propanol hydrochloride
trimethadionum trimethadione	3,5,5-trimethyloxazolidine-2,4-dione
tripelennamini hydrochloridum tripelennamine hydrochloride	N-benzyl-N,′N′-dimethyl-N-2-pyridylethylenediamine hydrochloride
tuaminoheptanum tuaminoheptane	1-methylhexylamine
tubocurarini chloridum tubocurarine chloride	*d*-tubocurarine chloride
tyrothricinum tyrothricin	
urethanum urethane	ethyl carbamate
vanyldisulfamidum vanyldisulfamide	4′-oxy-5′-methoxy-1-benzylidene-bis-(aminophenylsulfonamide)
vinbarbitalum vinbarbital	5-ethyl-5-(1-methyl-1-butenyl) barbituric acid

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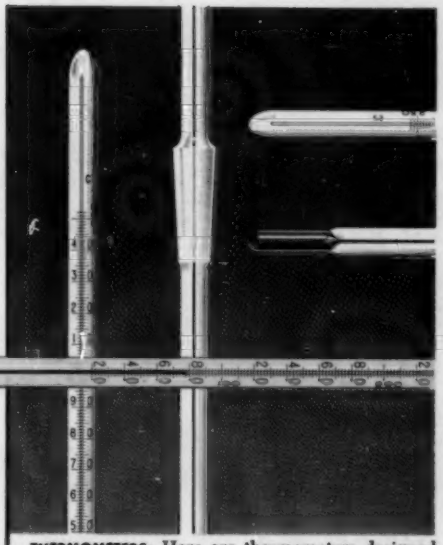

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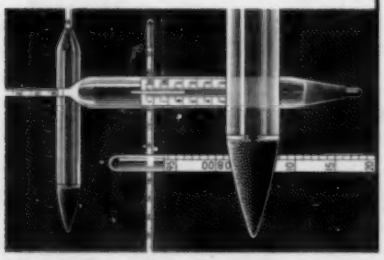

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